

BT

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
23 September 2004 (23.09.2004)

PCT

(10) International Publication Number
WO 2004/080425 A2

- (51) International Patent Classification⁷: **A61K**
- (21) International Application Number: **PCT/US2004/007755**
- (22) International Filing Date: 12 March 2004 (12.03.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/454,300 12 March 2003 (12.03.2003) US
60/454,432 12 March 2003 (12.03.2003) US
- (71) Applicant (for all designated States except US): **VAS-GENE THERAPEUTICS, INC.** [US/US]; 1929 Zonal Avenue, Los Angeles, CA 90033 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **KRASNOPEROV, Valery** [RU/US]; 346 South Los Robles Avenue, Apt.6, Pasadena, CA 91101 (US). **ZOZULYA, SERGEY** [RU/US]; 255 S. Grand Avenue, Apt. 2308, Los Angeles, CA 90012 (US). **KERETESZ, Nathalie** [CA/US]; 4240 Lost Hills Road, Apt 1603, Calabasas, CA 91301 (US). **REDDY, Ramachandra** [US/US]; 2101 Charlotte Street, Apt. 21, Los Angeles, LA 90033 (US). **GILL, Parkash** [US/US]; 1929 Zonal Avenue, Los Angeles, CA 90033 (US).
- (54) Title: **POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH**
- (57) Abstract: In certain embodiments, this present invention provides polypeptide compositions, and methods for inhibiting Ephrin B2 or EphB4 activity. In other embodiments, the present invention provides methods and compositions for treating cancer or for treating angiogenesis-associated diseases.
- (74) Agents: **VINCENT, Matthew, P. et al.**; Ropes & Gray LLP, One International Place, Boston, MA 02110-2624 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/080425 A2

BEST AVAILABLE COPY

POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

5 RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application number 60/454,300 filed March 12, 2003 and U.S. Provisional Application number 60/454,432 filed March 12, 2003. The entire teachings of the referenced Provisional Applications are incorporated herein by reference in their entirety.

10

BACKGROUND OF THE INVENTION

Angiogenesis, the development of new blood vessels from the endothelium of a preexisting vasculature, is a critical process in the growth, progression, and metastasis of solid tumors within the host. During physiologically normal angiogenesis, the autocrine, paracrine, and amphicrine interactions of the vascular endothelium with its surrounding stromal components are tightly regulated both spatially and temporally. Additionally, the levels and activities of proangiogenic and angiostatic cytokines and growth factors are maintained in balance. In contrast, the pathological angiogenesis necessary for active tumor growth is sustained and persistent, representing a dysregulation of the normal angiogenic system. Solid and hematopoietic tumor types are particularly associated with a high level of abnormal angiogenesis.

It is generally thought that the development of tumor consists of sequential, and interrelated steps that lead to the generation of an autonomous clone with aggressive growth potential. These steps include sustained growth and unlimited self-renewal. Cell populations in a tumor are generally characterized by growth signal self-sufficiency, decreased sensitivity to growth suppressive signals, and resistance to apoptosis. Genetic or cytogenetic events that initiate aberrant growth sustain cells in a prolonged "ready" state by preventing apoptosis.

It is a goal of the present disclosure to provide agents and therapeutic treatments for inhibiting angiogenesis and tumor growth.

30

SUMMARY OF THE INVENTION

In certain aspects, the disclosure provides polypeptide agents that inhibit EphB4 or EphrinB2 mediated functions, including monomeric ligand binding portions of the EphB4 and EphrinB2 proteins and antibodies that bind to and affect EphB4 or EphrinB2 in particular ways. As demonstrated herein, EphB4 and EphrinB2 participate in various disease states, including cancers and diseases related to unwanted or excessive angiogenesis. Accordingly, certain polypeptide agents disclosed herein may be used to treat such diseases. In further aspects, the disclosure relates to the discovery that EphB4 and/or EphrinB2 are expressed, often at high levels, in a variety of tumors. Therefore, polypeptide agents that downregulate EphB4 or EphrinB2 function may affect tumors by a direct effect on the tumor cells as well as an indirect effect on the angiogenic processes recruited by the tumor. In certain embodiments, the disclosure provides the identity of tumor types particularly suited to treatment with an agent that downregulates EphB4 or EphrinB2 function.

In certain aspects, the disclosure provides soluble EphB4 polypeptides comprising an amino acid sequence of an extracellular domain of an EphB4 protein. The soluble EphB4 polypeptides bind specifically to an EphrinB2 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphB4 for binding to ligand such as EphrinB2 and inhibit the signaling that results from EphB4 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. In certain embodiments the soluble EphB4 polypeptide comprises a globular domain of an EphB4 protein. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-522 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-412 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-312 of the amino acid sequence defined by Figure 65. A soluble EphB4 polypeptide may comprise a sequence as set forth in Figure 1 or 2. In certain embodiments, the soluble EphB4 polypeptide may inhibit the interaction between Ephrin B2 and EphB4. The soluble EphB4 polypeptide may inhibit clustering of or phosphorylation of Ephrin B2 or EphB4. Phosphorylation of EphrinB2 or EphB4 is generally considered to be one of the

initial events in triggering intracellular signaling pathways regulated by these proteins. As noted above, the soluble EphB4 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

In certain aspects, the disclosure provides soluble EphrinB2 polypeptides comprising an amino acid sequence of an extracellular domain of an EphrinB2 protein. The soluble EphrinB2 polypeptides bind specifically to an EphB4 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphrinB2 for binding to ligand such as EphB4 and inhibit the signaling that results from EphrinB2 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. A soluble EphrinB2 polypeptide may comprise residues 1-225 of the amino acid sequence defined by Figure 66. A soluble EphrinB2 polypeptide may comprise a sequence defined by Figure 3. In certain embodiments, the soluble EphrinB2 polypeptide may inhibit the interaction between Ephrin B2 and EphB4. The soluble EphrinB2 polypeptide may inhibit clustering of or phosphorylation of EphrinB2 or EphB4. As noted above, the soluble EphrinB2 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

In certain aspects, the disclosure provides antagonist antibodies for EphB4 and EphrinB2. An antibody may be designed to bind to an extracellular domain of an EphB4 protein and inhibit an activity of the EphB4. An antibody may be designed to bind to an extracellular domain of an Ephrin B2 protein and inhibit an activity of the Ephrin B2. An antibody may be designed to inhibit the interaction between Ephrin B2 and EphB4. An antagonist antibody will generally affect Eph and/or Ephrin signaling. For example, an antibody may inhibit clustering or phosphorylation of Ephrin B2 or EphB4. An antagonist antibody may be essentially any polypeptide comprising a variable portion of an antibody,

including, for example, monoclonal and polyclonal antibodies, single chain antibodies, diabodies, minibodies, etc.

In certain aspects, the disclosure provides pharmaceutical formulations comprising a polypeptide reagent and a pharmaceutically acceptable carrier. The polypeptide reagent may
5 be any disclosed herein, including, for example, soluble EphB4 or EphrinB2 polypeptides and antagonist antibodies. Additional formulations include cosmetic compositions and diagnostic kits.

In certain aspects the disclosure provides methods of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell. A method may comprise contacting the cell with an
10 effective amount of a polypeptide agent, such as (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity
15 to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; or (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects the disclosure provides methods for reducing the growth rate of a tumor, comprising administering an amount of a polypeptide agent sufficient to reduce the
20 growth rate of the tumor, wherein the polypeptide agent is selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a
25 monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. Optionally, the tumor comprises cells expressing a higher level of EphB4 and/or EphrinB2 than noncancerous cells of a comparable tissue.

30 In certain aspects, the disclosure provides methods for treating a patient suffering from a cancer. A method may comprise administering to the patient a polypeptide agent selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid

sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

Optionally, the cancer comprises cancer cells expressing EphrinB2 and/or EphB4 at a higher level than noncancerous cells of a comparable tissue. The cancer may be a metastatic cancer.

The cancer may be selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.

Optionally, the cancer is an angiogenesis-dependent cancer or an angiogenesis independent cancer. The polypeptide agent employed may inhibit clustering or phosphorylation of Ephrin B2 or EphB4. A polypeptide agent may be co-administered with one or more additional anti-cancer chemotherapeutic agents that inhibit cancer cells in an additive or synergistic manner with the polypeptide agent.

In certain aspects, the disclosure provides methods of inhibiting angiogenesis. A method may comprise contacting a cell with an amount of a polypeptide agent sufficient to inhibit angiogenesis, wherein the polypeptide agent is selected from the group consisting of:

(a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects, the disclosure provides methods for treating a patient suffering from an angiogenesis-associated disease, comprising administering to the patient a

polypeptide agent selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2

protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2. The soluble polypeptide may be formulated with a pharmaceutically acceptable carrier. An angiogenesis related disease or unwanted angiogenesis related process may be selected from the group consisting of angiogenesis-dependent cancer, benign tumors, inflammatory disorders, chronic articular rheumatism and psoriasis, ocular angiogenic diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, wound granulation, wound healing, telangiectasia psoriasis scleroderma, pyogenic granuloma, coronary collaterals, ischemic limb angiogenesis, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, and hematopoiesis. An polypeptide agent may be co-administered with at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the soluble polypeptide.

In certain aspects, the disclosure provides for the use of a polypeptide agent in the manufacture of medicament for the treatment of cancer or an angiogenesis related disorder, wherein the polypeptide agent is selected from the group consisting of: (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

In certain aspects, the disclosure provides methods for for treating a patient suffering from a cancer, comprising: (a) identifying in the patient a tumor having a plurality of cancer cells that express EphB4 and/or EphrinB2; and (b) administering to the patient a polypeptide agent selected from the group consisting of: (i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; (ii) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein,

wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide; (iii) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and (iv) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

- 5 Optionally, a method may comprise identifying in the patient a tumor having a plurality of cancer cells having a gene amplification of the EphB4 and/or EphrinB2 gene.

In certain aspects, the disclosure provides methods for identifying a tumor that is suitable for treatment with an EphrinB2 or EphB4 antagonist. A method may comprise detecting in the tumor cell one or more of the following characteristics: (a) expression of
10 EphB4 protein and/or mRNA; (b) expression of EphrinB2 protein and/or mRNA; (c) gene amplification of the EphB4 gene; or (d) gene amplification of the EphrinB2 gene. A tumor cell having one or more of characteristics (a)-(d) may be suitable for treatment with an EphrinB2 or EphB4 antagonist, such as a polypeptide agent described herein.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows amino acid sequence of the B4ECv3 protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 2 shows amino acid sequence of the B4ECv3NT protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

- 20 Figure 3 shows amino acid sequence of the B2EC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

Figure 4 shows amino acid sequence of the B4ECv3-FC protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

- 25 Figure 5 shows amino acid sequence of the B2EC-FC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

Figure 6 shows B4EC-FC binding assay (Protein A-agarose based).

Figure 7 shows B4EC-FC inhibition assay (Inhibition in solution).

Figure 8 shows B2EC-FC binding assay (Protein-A-agarose based assay).

Figure 9 shows chemotaxis of HUAEC in response to B4Ecv3.

- 30 Figure 10 shows chemotaxis of HHEC in response to B2EC-FC.

Figure 11 shows chemotaxis of HHAEC in response to B2EC.

Figure 12 shows effect of B4Ecv3 on HUAEC tubule formation.

Figure 13 shows effect of B2EC-FC on HUAEC tubule formation.

Figure 14 is a schematic representation of human Ephrin B2 constructs.

5 Figure 15 is a schematic representation of human EphB4 constructs.

Figure 16 shows the domain structure of the recombinant soluble EphB4EC proteins. Designation of the domains are as follows: L - leader peptide, G - globular (ligand-binding domain), C - Cys-rich domain, F1, F2 - fibronectin type III repeats, H - 6 x His-tag.

Figure 17 shows purification and ligand binding properties of the EphB4EC proteins.
10 A. SDS-PAAG gel electrophoresis of purified EphB4-derived recombinant soluble proteins (Coomassie-stained). B. Binding of Ephrin B2-AP fusion to EphB4-derived recombinant proteins immobilized on Ni-NTA-agarose beads. Results of three independent experiments are shown for each protein. Vertical axis - optical density at 420 nm.

Figure 18 shows that EphB4v3 inhibits chemotaxis.

15 Figure 19 shows that EphB4v3 inhibits tubule formation on Matrigel. A displays the strong inhibition of tubule formation by B4v3 in a representative experiment. B shows a quantitation of the reduction of tube-length obtained with B4v3 at increasing concentrations as well as a reduction in the number of junctions, in comparison to cells with no protein. Results are displayed as mean values \pm S.D. obtained from three independent experiments
20 performed with duplicate wells.

Figure 20 shows that soluble EphB4 has no detectable cytotoxic effect as assessed by MTS assay.

Figure 21 shows that B4v3 inhibits invasion and tubule formation by endothelial cells in the Matrigel assay. (A) to detect total invading cells, photographed at 20X magnification
25 or with Masson's Trichrome Top left of A B displays section of a Matrigel plug with no GF, *top right* of A displays section with B4IgG containing GF and *lower left* section contains GF, and lower right shows GF in the presence of B4v3. Significant invasion of endothelial cells is only seen in GF containing Matrigel. *Top right* displays an area with a high number of invaded cells induced by B4IgG, which signifies the dimeric form of B4v3. The *left upper*
30 *parts* of the pictures correspond to the cell layers formed around the Matrigel plug from which cells invade toward the center of the plug located in the direction of the *right lower*

corner. Total cells in sections of the Matrigel plugs were quantitated with Scion Image software. Results obtained from two experiments with duplicate plugs are displayed as mean values \pm S.D.

5 Figure 22 shows tyrosine phosphorylation of EphB4 receptor in PC3 cells in response to stimulation with EphrinB2-Fc fusion in presence or absence of EphB4-derived recombinant soluble proteins.

Figure 23 shows effects of soluble EphB4ECD on viability and cell cycle. A) 3-day cell viability assay of two HNSCC cell lines. B) FACS analysis of cell cycle in HNSCC-15 cells treated as in A. Treatment of these cells resulted in accumulation in subG0/G1 and S/G2
10 phases as indicated by the arrows.

Figure 24 shows that B4v3 inhibits neovascular response in a murine corneal hydropic micropocket assay.

Figure 25 shows that that SCC15, B16, and MCF-7 co-injected with sB4v3 in the presence of matrigel and growth factors, inhibits the in vivo tumor growth of these cells.

15 Figure 26 shows that soluble EphB4 causes apoptosis, necrosis and decreased angiogenesis in three tumor types, B16 melanoma, SCC15, head and neck carcinoma, and MCF-7 Breast carcinoma. Tumors were injected premixed with Matrigel plus growth factors and soluble EphB4 subcutaneously. After 10 to 14 days, the mice were injected intravenously with FITC-lectin (green) to assess blood vessel perfusion. Tumors treated with
20 control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with sEphB4 displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 27 shows expression of EphB4 in prostate cell lines. A) Western blot of total
25 cell lysates of various prostate cancer cell lines, normal prostate gland derived cell line (MLC) and acute myeloblastic lymphoma cells (AML) probed with EphB4 monoclonal antibody. B) Phosphorylation of EphB4 in PC-3 cells determined by Western blot.

Figure 28 shows expression of EphB4 in prostate cancer tissue. Representative prostate cancer frozen section stained with EphB4 monoclonal antibody (top left) or isotype specific control (bottom left). Adjacent BPH tissue stained with EphB4 monoclonal antibody
30 (top right). Positive signal is brown color in the tumor cells. Stroma and the normal epithelia are negative. Note membrane localization of stain in the tumor tissue, consistent with trans-

membrane localization of EphB4. Representative QRT-PCR of RNA extracted from cancer specimens and adjacent BPH tissues (lower right).

Figure 29 shows downregulation of EphB4 in prostate cancer cells by tumor suppressors and RXR expression. A) PC3 cells were co-transfected with truncated CD4 and p53 or PTEN or vector only. 24 h later CD4-sorted cells were collected, lysed and analyzed sequentially by Western blot for the expression of EphB4 and β -actin, as a normalizer protein. B) Western blot as in (A) of various stable cell lines. LNCaP-FGF is a stable transfection clone of FGF-8, while CWR22R-RXR stably expresses the RXR receptor. BPH-1 was established from benign hypertrophic prostatic epithelium.

Figure 30 shows downregulation of EphB4 in prostate cancer cells by EGFR and IGFR-1. A) Western blot of PC3 cells treated with or without EGFR specific inhibitor AG1478 (1 nM) for 36 hours. Decreased EphB4 signal is observed after AG 1478 treatment. The membrane was stripped and reprobed with β -actin, which was unaffected. B) Western Blot of triplicate samples of PC3 cells treated with or without IGFR-1 specific neutralizing antibody MAB391 (2 μ g/ml; overnight). The membrane was sequentially probed with EphB4, IGFR-1 and β -actin antibodies. IGFR-1 signal shows the expected repression of signal with MAB391 treatment.

Figure 31 shows effect of specific EphB4 AS-ODNs and siRNA on expression and prostate cell functions. A) 293 cells stably expressing full-length construct of EphB4 was used to evaluate the ability of siRNA 472 to inhibit EphB4 expression. Cells were transfected with 50 nM RNAi using Lipofectamine 2000. Western blot of cell lysates 40 h post transfection with control siRNA (green fluorescence protein; GFP siRNA) or EphB4 siRNA 472, probed with EphB4 monoclonal antibody, stripped and reprobed with β -actin monoclonal antibody. B) Effect of EphB4 AS-10 on expression in 293 transiently expressing full-length EphB4. Cells were exposed to AS-10 or sense ODN for 6 hours and analyzed by Western blot as in (A). C) 48 h viability assay of PC3 cells treated with siRNA as described in the Methods section. Shown is mean \pm s.e.m. of triplicate samples. D) 5-day viability assay of PC3 cells treated with ODNs as described in the Methods. Shown is mean \pm s.e.m. of triplicate samples. E) Scrape assay of migration of PC3 cells in the presence of 50 nM siRNAs transfected as in (A). Shown are photomicrographs of representative 20x fields taken immediately after the scrape was made in the monolayer (0 h) and after 20h continued culture. A large number of cells have filled in the scrape after 20 h with control siRNA, but not with EphB4 siRNA 472. F) Shown is a similar assay for cells treated with AS-10 or sense

ODN (both 10 μ M). G) Matrigel invasion assay of PC3 cells transfected with siRNA or control siRNA as described in the methods. Cells migrating to the underside of the Matrigel coated insert in response to 5 mg/ml fibronectin in the lower chamber were fixed and stained with Giemsa. Shown are representative photomicrographs of control siRNA and siRNA 472 treated cells. Cell numbers were counted in 5 individual high-powered fields and the average \pm s.e.m. is shown in the graph (bottom right).

Figure 32 shows effect of EphB4 siRNA 472 on cell cycle and apoptosis. A) PC3 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. 7.9% of the cell population is apoptotic (in the Sub G0 peak) when treated with siRNA 472 compared to 1% with control siRNA. B) Apoptosis of PC3 cells detected by Cell Death Detection ELISA^{plus} kit as described in the Methods. Absorbance at 405 nm increases in proportion to the amount of histone and DNA-POD in the nuclei-free cell fraction. Shown is the mean \pm s.e.m. of triplicate samples at the indicated concentrations of siRNA 472 and GFP siRNA (control).

Figure 33 shows that EphB4 and EphrinB2 are expressed in mesothelioma cell lines as shown by RT-PCR (A) and Western Blot (B).

Figure 34 shows expression of ephrin B2 and EphB4 by in situ hybridization in mesothelioma cells. NCI H28 mesothelioma cell lines cultured in chamber slides hybridized with antisense probe to ephrin B2 or EphB4 (top row). Control for each hybridization was sense (bottom row). Positive reaction is dark blue cytoplasmic stain.

Figure 35 shows cellular expression of EphB4 and ephrin B2 in mesothelioma cultures. Immunofluorescence staining of primary cell isolate derived from pleural effusion of a patient with malignant mesothelioma and cell lines NCI H28, NCI H2373, and NCI H2052 for ephrin B2 and EphB4. Green color is positive signal for FITC labeled secondary antibody. Specificity of immunofluorescence staining was demonstrated by lack of signal with no primary antibody (first row). Cell nuclei were counterstained with DAPI (blue color) to reveal location of all cells. Shown are merged images of DAPI and FITC fluorescence. Original magnification 200X.

Figure 36 shows expression of ephrin B2 and EphB4 in mesothelioma tumor. Immunohistochemistry of malignant mesothelioma biopsy. H&E stained section to reveals

tumor architecture; bottom left panel is background control with no primary antibody. EphB4 and ephrin B2 specific staining is brown color. Original magnification 200X.

Figure 37 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on the growth of H28 cells.

5 Figure 38 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on cell migration.

Figure 39 shows that EphB4 is expressed in HNSCC primary tissues and metastases.

A) Top: Immunohistochemistry of a representative archival section stained with EphB4 monoclonal antibody as described in the methods and visualized with DAB (brown color) localized to tumor cells. Bottom: Hematoxylin and Eosin (H&E) stain of an adjacent section. Dense purple staining indicates the presence of tumor cells. The right hand column are frozen sections of lymph node metastasis stained with EphB4 polyclonal antibody (top right) and visualized with DAB. Control (middle) was incubation with goat serum and H&E (bottom) reveals the location of the metastatic foci surrounded by stroma which does not stain. B) In situ hybridization of serial frozen sections of a HNSCC case probed with EphB4 (left column) and ephrin B2 (right column) DIG labeled antisense or sense probes generated by run-off transcription. Hybridization signal (dark blue) was detected using alkaline-phosphatase-conjugated anti-DIG antibodies and sections were counterstained with Nuclear Fast Red. A serial section stained with H&E is shown (bottom left) to illustrate tumor architecture. C) Western blot of protein extract of patient samples consisting of tumor (T), uninvolved normal tissue (N) and lymph node biopsies (LN). Samples were fractionated by polyacrylamide gel electrophoresis in 4-20% Tris-glycine gels and subsequently electroblotted onto nylon membranes. Membranes were sequentially probed with EphB4 monoclonal antibody and β -actin MoAb. Chemiluminescent signal was detected on

10
15
20
25

autoradiography film. Shown is the EphB4 specific band which migrated at 120 kD and β -actin which migrated at 40 kD. The β -actin signal was used to control for loading and transfer of each sample.

Figure 40 shows that EphB4 is expressed in HNSCC cell lines and is regulated by EGF: A) Survey of EphB4 expression in SCC cell lines. Western blot of total cell lysates sequentially probed with EphB4 monoclonal antibody, stripped and reprobed with β -actin monoclonal antibody as described for Fig. 39C. B) Effect of the specific EGFR inhibitor AG1478 on EphB4 expression: Western blot of crude cell lysates of SCC15 treated with 0-

30

1000 nM AG 1478 for 24 h in media supplemented with 10% FCS (left) or with 1 mM AG 1478 for 4, 8, 12 or 24 h (right). Shown are membranes sequentially probed for EphB4 and β -actin. C) Effect of inhibition of EGFR signaling on EphB4 expression in SCC cell lines: Cells maintained in growth media containing 10% FCS were treated for 24 hr with 1 μ M AG 1478, after which crude cell lysates were analyzed by Western blots of cell lysates sequentially probed with for EGFR, EphB4, ephrin B2 and β -actin antibodies. Specific signal for EGFR was detected at 170 kD and ephrin B2 at 37 kD in addition to EphB4 and β -actin as described in Fig. 1C. β -actin serves as loading and transfer control.

Figure 41 shows mechanism of regulation of EphB4 by EGF: A) Schematic of the EGFR signaling pathways, showing in red the sites of action and names of specific kinase inhibitors used. B) SCC15 cells were serum-starved for 24 h prior to an additional 24 incubation as indicated with or without EGF (10 ng/ml), 3 μ M U73122, or 5 μ M SH-5, 5 μ M SP600125, 25 nM LY294002, -- μ M PD098095 or 5 μ M SB203580. N/A indicates cultures that received equal volume of diluent (DMSO) only. Cell lysates were subjected to Western Blot with EphB4 monoclonal antibody. β -actin signal serves as control of protein loading and transfer.

Figure 42 shows that specific EphB4 siRNAs inhibit EphB4 expression, cell viability and cause cell cycle arrest. A) 293 cells stably expressing full length EphB4 were transfected with 50 nM RNAi using LipofectamineTM2000. 40 h post-transfection cells were harvested, lysed and processed for Western blot. Membranes were probed with EphB4 monoclonal antibody, stripped and reprobed with β -actin monoclonal antibody as control for protein loading and transfer. Negative reagent control was RNAi to scrambled green fluorescence protein (GFP) sequence and control is transfection with LipofectamineTM2000 alone. B) MTT cell viability assays of SCC cell lines treated with siRNAs for 48 h as described in the Methods section. Shown is mean + s.e.m. of triplicate samples. C) SCC15 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. Top and middle row show plots for cells 16 h after siRNA transfection, bottom row shows plots for cells 36 h post transfection. Specific siRNA and concentration are indicated for each plot. Lipo = LipofectamineTM2000 mock transfection.

Figure 43 shows in vitro effects of specific EphB4 AS-ODNs on SCC cells. A) 293 cells transiently transfected with EphB4 full-length expression plasmid were treated 6 h post transfection with antisense ODNs as indicated. Cell lysates were collected 24 h after AS-

ODN treatment and subjected to Western Blot. B) SCC25 cells were seeded on 48 well plates at equal densities and treated with EphB4 AS-ODNs at 1, 5, and 10 μ M on days 2 and 4. Cell viability was measured by MTT assay on day 5. Shown is the mean + s.e.m. of triplicate samples. Note that AS-ODNs that were active in inhibiting EphB4 protein levels were also effective inhibitors of SCC15 cell viability. C) Cell cycle analysis of SCC15 cells treated for 36 h with AS-10 (bottom) compared to cells that were not treated (top). D) Confluent cultures of SCC15 cells scraped with a plastic Pasteur pipette to produce 3 mm wide breaks in the monolayer. The ability of the cells to migrate and close the wound in the presence of inhibiting EphB4 AS-ODN (AS-10) and non-inhibiting AS-ODN (AS-1) was assessed after 48 h. Scrambled ODN is included as a negative control ODN. Culture labeled no treatment was not exposed to ODN. At initiation of the experiment, all cultures showed scrapes of equal width and similar to that seen in 1 μ M EphB4 AS-10 after 48 h. The red brackets indicate the width of the original scrape. E) Migration of SCC15 cells in response to 20 mg/ml EGF in two-chamber assay as described in the Methods. Shown are representative photomicrographs of non-treated (NT), AS-6 and AS-10 treated cells and 10 ng/ml Taxol as positive control of migration inhibition. F) Cell numbers were counted in 5 individual high-powered fields and the average + s.e.m. is shown in the graph.

Figure 44 shows that EphB4 AS-ODN inhibits tumor growth in vivo. Growth curves for SCC15 subcutaneous tumor xenografts in Balb/C nude mice treated with EphB4 AS-10 or scrambled ODN at 20 mg/kg/day starting the day following implantation of 5×10^6 cells. Control mice received an equal volume of diluent (PBS). Shown are the mean + s.e.m. of 6 mice/group. * $P = 0.0001$ by Student's t-test compared to scrambled ODN treated group.

Figure 45 shows that Ephrin B2, but not EphB4 is expressed in KS biopsy tissue. (A) In situ hybridization with antisense probes for ephrin B2 and EphB4 with corresponding H&E stained section to show tumor architecture. Dark blue color in the ISH indicates positive reaction for ephrin B2. No signal for EphB4 was detected in the Kaposi's sarcoma biopsy. For contrast, ISH signal for EphB4 is strong in squamous cell carcinoma tumor cells. Ephrin B2 was also detected in KS using EphB4-AP fusion protein (bottom left). (B) Detection of ephrin B2 with EphB4/Fc fusion protein. Adjacent sections were stained with H&E (left) to show tumor architecture, black rectangle indicates the area shown in the EphB4/Fc treated section (middle) detected with FITC-labeled anti-human Fc antibody as described in the methods section. As a control an adjacent section was treated with human Fc fragment (right). Specific signal arising from EphB4/Fc binding to the section is seen only in

areas of tumor cells. (C) Co-expression of ephrin B2 and the HHV8 latency protein LANA1. Double-label confocal immunofluorescence microscopy with antibodies to ephrin B2 (red) LANA1 (green), or EphB4 (red) of frozen KS biopsy material directly demonstrates co-expression of LANA1 and ephrin B2 in KS biopsy. Coexpression is seen as yellow color.

- 5 Double label confocal image of biopsy with antibodies to PECAM-1 (green) in cells with nuclear propidium iodide stain (red), demonstrating the vascular nature of the tumor.

Figure 46 shows that HHV-8 induces arterial marker expression in venous endothelial cells. (A) Immunofluorescence of cultures of HUVEC and HUVEC/BC-1 for artery/vein markers and viral proteins. Cultures were grown on chamber slides and processed
10 for immunofluorescence detection of ephrin B2 (a, e, i), EphB4 (m, q, u), CD148 (j, v), and the HHV-8 proteins LANA1 (b, f, m) or ORF59 (r) as described in the Materials and Methods. Yellow color in the merged images of the same field demonstrate co-expression of ephrin B2 and LANA or ephrin B2 and CD148. The positions of viable cells were revealed
15 by nuclear staining with DAPI (blue) in the third column (c, g, k, o, s, w). Photomicrographs are of representative fields. (B) RT-PCR of HUVEC and two HHV-8 infected cultures (HUVEC/BC-1 and HUVEC/BC-3) for ephrin B2 and EphB4. Ephrin B2 product (200 bp) is seen in HUVEC/BC-1, HUVEC/BC-3 and EphB4 product (400 bp) is seen in HUVEC. Shown also is β -actin RT-PCR as a control for amount and integrity of input RNA.

Figure 47 shows that HHV-8 induces arterial marker expression in Kaposi's sarcoma
20 cells. (A) Western blot for ephrin B2 on various cell lysates. SLK-vGPCR is a stable clone of SLK expressing the HHV-8 vGPCR, and SLK-pCEFL is control stable clone transfected with empty expression vector. SLK cells transfected with LANA or LANA Δ 440 are SLK-LANA and SLK- Δ 440 respectively. Quantity of protein loading and transfer was determined by reprobing the membranes with β -actin monoclonal antibody. (B) Transient transfection of
25 KS-SLK cells with expression vector pvGPCR-CEFL resulted in the expression of ephrin B2 as shown by immunofluorescence staining with FITC (green), whereas the control vector pCEFL had no effect. KS-SLK cells (0.8×10^5 /well) were transfected with $0.8 \mu\text{g}$ DNA using Lipofectamine 2000. 24 hr later cells were fixed and stained with ephrin B2 polyclonal antibody and FITC conjugated secondary antibody as described in the methods. (C) Transient
30 transfection of HUVEC with vGPCR induces transcription from ephrin B2 luciferase constructs. 8×10^3 HUVEC in 24 well plates were transfected using Superfect with $0.8 \mu\text{g}$ /well ephrin B2 promoter constructs containing sequences from -2941 to -11 with respect to the translation start site, or two 5'-deletions as indicated, together with 80 ng/well pCEFL

or pvGPCR-CEFL. Luciferase was determined 48 h post transfection and induction ratios are shown to the right of the graph. pGL3Basic is promoterless luciferase control vector.

Luciferase was normalized to protein since GPCR induced expression of the cotransfected β -galactosidase. Graphed is mean + SEM of 6 replicates. Shown is one of three similar

5 experiments.

Figure 48 shows that VEGF and VEGF-C regulate ephrin B2 expression. A) Inhibition of ephrin B2 by neutralizing antibodies. Cells were cultured in full growth medium and exposed to antibody (100 ng/ml) for 36 hr before collection and lysis for Western blot. B) For induction of ephrin B2 expression cells were cultured in EBM growth medium containing
10 5% serum lacking growth factors. Individual growth factors were added as indicated and the cells harvested after 36 h. Quantity of protein loading and transfer was determined by reprobing the membranes β -actin monoclonal antibody.

Figure 49 shows that Ephrin B2 knock-down with specific siRNA inhibits viability in KS cells and HUVEC grown in the presence of VEGF but not IGF, EGF or bFGF. A) KS-
15 SLK cells were transfected with various siRNA to ephrin B2 and controls. After 48 hr the cells were harvested and crude cell lysates fractionated on 4-20% SDS-PAGE. Western blot was performed with monoclonal antibody to ephrin B2 generated in-house. The membrane was stripped and reprobed with β -actin monoclonal antibody (Sigma) to illustrate equivalent loading and transfer. B) 3 day cell viability assay of KS-SLK cultures in the presence of
20 ephrin B2 and EphB4 siRNAs. 1×10^5 cells/well in 24-well plates were treated with 0, 10 and 100 ng/ml siRNAs as indicated on the graph. Viability of cultures was determined by MTT assay as described in the methods section. Shown are the mean + standard deviation of duplicate samples. C) HUVE cells were seeded on eight wells chamber slides coated with fibronectin. The HUVE cells were grown overnight in EGM-2 media, which contains all
25 growth supplements. On the following day, the media was replaced with media containing VEGF (10ng/ml) or EGF, FGF and IGF as indicated. After 2 hrs of incubation at 37 °C, the cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium containing 10 nM of siRNA to ephrin B2, Eph B4 or green fluorescence protein (GFP) as control. The cells were incubated for 2 hr and then the fresh media containing growth factors
30 or VEGF alone was added to their respective wells. After 48 hrs, the cells were stained with crystal violet and the pictures were taken immediately by digital camera at 10X magnification.

Figure 50 shows that soluble EphB4 inhibits KS and EC cord formation and in vivo angiogenesis. Cord formation assay of HUVEC in MatrigelTM (upper row). Cells in exponential growth phase were treated overnight with the indicated concentrations of EphB4 extracellular domain (ECD) prior to plating on MatrigelTM. Cells were trypsinized and plated (1×10^5 cells/well) in a 24-well plate containing 0.5 ml MatrigelTM. Shown are representative 20X phase contrast fields of cord formation after 8 hr plating on MatrigelTM in the continued presence of the test compounds as shown. Original magnification 200 X. KS-SLK cells treated in a similar manner (middle row) in a cord formation assay on MatrigelTM. Bottom row shows in vivo MatrigelTM assay: MatrigelTM plugs containing growth factors and EphB4 ECD or PBS were implanted subcutaneously in the mid-ventral region of mice. After 7 days the plugs were removed, sectioned and stained with H&E to visualize cells migrating into the matrix. Intact vessels with large lumens are observed in the control, whereas EphB4 ECD almost completely inhibited migration of cells into the Matrigel.

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μ M).

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

Figure 57 shows comparison of EphB4 monoclonal antibodies by G250 and in pull-down assay.

Figure 58 shows that EphB4 antibodies inhibit the growth of SCC15 xenograft tumors.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Figure 60 shows that systemic administration of EphB4 antibodies leads to tumor regression.

5 Figure 61 shows a genomic nucleotide sequence of human EphB4.

Figure 62 shows a cDNA nucleotide sequence of human EphB4.

Figure 63 shows a genomic nucleotide sequence of human Ephrin B2.

Figure 64 shows a cDNA nucleotide sequence of human Ephrin B2.

Figure 65 shows an amino acid sequence of human EphB4.

10 Figure 66 shows an amino acid sequence of human Ephrin B2.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

15 The current invention is based in part on the discovery that signaling through the ephrin/ephrin receptor pathway contributes to tumorigenesis. Applicants detected expression of ephrin B2 and EphB4 in tumor tissues and developed anti-tumor therapeutic agents for blocking signaling through the ephrin/ephrin receptor. In addition, the disclosure provides polypeptide therapeutic agents and methods for polypeptide-based inhibition of the function of EphB4 and/or Ephrin B2. Accordingly, in certain aspects, the disclosure provides
20 numerous polypeptide compounds (agents) that may be used to treat cancer as well as angiogenesis related disorders and unwanted angiogenesis related processes.

As used herein, the terms Ephrin and Eph are used to refer, respectively, to ligands and receptors. They can be from any of a variety of animals (e.g., mammals/non-mammals, vertebrates/non-vertebrates, including humans). The nomenclature in this area has changed
25 rapidly and the terminology used herein is that proposed as a result of work by the Eph Nomenclature Committee, which can be accessed, along with previously-used names at web site <http://www.eph-nomenclature.com>.

The work described herein, particularly in the examples, refers to Ephrin B2 and EphB4. However, the present invention contemplates any ephrin ligand and/or Eph receptor
30 within their respective family, which is expressed in a tumor. The ephrins (ligands) are of

two structural types, which can be further subdivided on the basis of sequence relationships and, functionally, on the basis of the preferential binding they exhibit for two corresponding receptor subgroups. Structurally, there are two types of ephrins: those which are membrane-anchored by a glycerophosphatidylinositol (GPI) linkage and those anchored through a transmembrane domain. Conventionally, the ligands are divided into the Ephrin-A subclass, which are GPI-linked proteins which bind preferentially to EphA receptors, and the Ephrin-B subclass, which are transmembrane proteins which generally bind preferentially to EphB receptors.

The Eph family receptors are a family of receptor protein-tyrosine kinases which are related to Eph, a receptor named for its expression in an erythropoietin-producing human hepatocellular carcinoma cell line. They are divided into two subgroups on the basis of the relatedness of their extracellular domain sequences and their ability to bind preferentially to Ephrin-A proteins or Ephrin-B proteins. Receptors which interact preferentially with Ephrin-A proteins are EphA receptors and those which interact preferentially with Ephrin-B proteins are EphB receptors.

Eph receptors have an extracellular domain composed of the ligand-binding globular domain, a cysteine rich region followed by a pair of fibronectin type III repeats (e.g., see Figure 16). The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α -motif (SAM) and a PDZ-domain binding motif. EphB4 is specific for the membrane-bound ligand Ephrin B2 (Sakano, S. et al 1996; Brambilla R. et al 1995). Ephrin B2 belongs to the class of Eph ligands that have a transmembrane domain and cytoplasmic region with five conserved tyrosine residues and PDZ domain. Eph receptors are activated by binding of clustered, membrane attached ephrins (Davis S et al, 1994), indicating that contact between cells expressing the receptors and cells expressing the ligands is required for Eph activation.

Upon ligand binding, an Eph receptor dimerizes and autophosphorylate the juxtamembrane tyrosine residues to acquire full activation (Kalo MS et al, 1999, Binns KS, 2000). In addition to forward signaling through the Eph receptor, reverse signaling can occur through the ephrin Bs. Eph engagement of ephrins results in rapid phosphorylation of the conserved intracellular tyrosines (Bruckner K, 1997) and somewhat slower recruitment of PDZ binding proteins (Palmer A 2002). Recently, several studies have shown that high expression of Eph/ephrins may be associated with increased potentials for tumor growth,

tumorigenicity, and metastasis (Easty DJ, 1999; Kiyokawa E, 1994; Tang XX, 1999; Vogt T, 1998; Liu W, 2002; Stephenson SA, 2001; Steube KG 1999; Berclaz G, 1996).

In certain embodiments, the present invention provides polypeptide therapeutic agents that inhibit activity of Ephrin B2, EphB4, or both. As used herein, the term "polypeptide therapeutic agent" or "polypeptide agent" is a generic term which includes any polypeptide that blocks signaling through the Ephrin B2/EphB4 pathway. A preferred polypeptide therapeutic agent of the invention is a soluble polypeptide of Ephrin B2 or EphB4. Another preferred polypeptide therapeutic agent of the invention is an antagonist antibody that binds to Ephrin B2 or EphB4. For example, such polypeptide therapeutic agent can inhibit function of Ephrin B2 or EphB4, inhibit the interaction between Ephrin B2 and EphB4, inhibit the phosphorylation of Ephrin B2 or EphB4, or inhibit any of the downstream signaling events upon binding of Ephrin B2 to EphB4.

II. Soluble Polypeptides

In certain aspects, the invention relates to a soluble polypeptide comprising an extracellular domain of an Ephrin B2 protein (referred to herein as an Ephrin B2 soluble polypeptide) or comprising an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide). Preferably, the subject soluble polypeptide is a monomer and is capable of binding with high affinity to Ephrin B2 or EphB4. In a specific embodiment, the EphB4 soluble polypeptide of the invention comprises a globular domain of an EphB4 protein. Specific examples EphB4 soluble polypeptides are provided in Figures 1, 2, and 15. Specific examples of Ephrin B2 soluble polypeptides are provided in Figures 3 and 14.

As used herein, the subject soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide or an Ephrin B2 soluble polypeptide. These fragments, functional variants, and modified forms of the subject soluble polypeptides antagonize function of EphB4, Ephrin B2 or both.

In certain embodiments, isolated fragments of the subject soluble polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding an EphB4 or Ephrin B2 soluble polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl

fragments that can function to inhibit function of EphB4 or Ephrin B2, for example, by testing the ability of the fragments to inhibit angiogenesis or tumor growth.

In certain embodiments, a functional variant of an EphB4 soluble polypeptide has an amino acid sequence that is at least 90%, 95%, 97%, 99% or 100% identical to residues 1-522, residues 1-412, or residues 1-312 of the amino acid sequence defined by Figure 65. In other embodiments, a functional variant of an Ephrin B2 soluble polypeptide has a sequence at least 90%, 95%, 97%, 99% or 100% identical to residues 1-225 of the amino acid sequence defined by Figure 66.

In certain embodiments, the present invention contemplates making functional variants by modifying the structure of the subject soluble polypeptide for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified soluble polypeptide are considered functional equivalents of the naturally-occurring EphB4 or Ephrin B2 soluble polypeptide. Modified soluble polypeptides can be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains.

This invention further contemplates a method of generating sets of combinatorial mutants of the EphB4 or Ephrin B2 soluble polypeptides, as well as truncation mutants, and is especially useful for identifying functional variant sequences. The purpose of screening such combinatorial libraries may be to generate, for example, soluble polypeptide variants which can act as antagonists of EphB4, EphB2, or both. Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring soluble polypeptide. Such variant proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding wild-type soluble polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the protein of interest (e.g., a soluble polypeptide). Such variants, and the genes which encode them, can be utilized to alter the subject soluble polypeptide

levels by modulating their half-life. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant soluble polypeptide levels within the cell. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential soluble polypeptide sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al., (1981) *Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al., (1984) *Science* 198:1056; Ike et al., (1983) *Nucleic Acid Res.* 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) *Science* 249:386-390; Roberts et al., (1992) *PNAS USA* 89:2429-2433; Devlin et al., (1990) *Science* 249: 404-406; Cwirla et al., (1990) *PNAS USA* 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, soluble polypeptide variants (e.g., the antagonist forms) can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) *Biochemistry* 33:1565-1572; Wang et al., (1994) *J. Biol. Chem.* 269:3095-3099; Balint et al., (1993) *Gene* 137:109-118; Grodberg et al., (1993) *Eur. J. Biochem.* 218:597-601; Nagashima et al., (1993) *J. Biol. Chem.* 268:2888-2892; Lowman et al., (1991) *Biochemistry* 30:10832-10838; and Cunningham et al., (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) *Virology* 193:653-660; Brown et al., (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight et al., (1982) *Science* 232:316); by saturation mutagenesis (Meyers et al., (1986) *Science* 232:613); by PCR mutagenesis (Leung et al., (1989) *Method Cell Mol Biol* 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) *A Short Course in Bacterial Genetics*, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) *Strategies in Mol Biol* 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial

setting, is an attractive method for identifying truncated (bioactive) forms of the subject soluble polypeptide.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of the subject soluble polypeptides. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In certain embodiments, the subject soluble polypeptides of the invention include a small molecule such as a peptide and a peptidomimetic. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that contain non-naturally occurring amino acids, peptoids, and the like. Peptidomimetics provide various advantages over a peptide, including enhanced stability when administered to a subject. Methods for identifying a peptidomimetic are well known in the art and include the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). Where no crystal structure of a target molecule is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro Calif.), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of the EphB4 or Ephrin B2 soluble polypeptides.

To illustrate, by employing scanning mutagenesis to map the amino acid residues of a soluble polypeptidewhich are involved in binding to another protein, peptidomimetic compounds can be generated which mimic those residues involved in binding. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine

(e.g., see Freidinger et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) *J. Med. Chem.* 29:295; and Ewenson et al., in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al., (1985) *Tetrahedron Lett* 26:647; and Sato et al., (1986) *J Chem Soc Perkin Trans 1*:1231), and b-aminoalcohols (Gordon et al., (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al., (1986) *Biochem Biophys Res Commun* 134:71).

In certain embodiments, the soluble polypeptides of the invention may further comprise post-translational modifications. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified soluble polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a soluble polypeptide may be tested for its antagonizing role in EphB4 or Ephrin B2 function, e.g, its inhibitory effect on angiogenesis or on tumor growth.

In certain aspects, functional variants or modified forms of the subject soluble polypeptides include fusion proteins having at least a portion of the soluble polypeptide and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, and an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), which are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt- conjugated resins are used. Another fusion domain well known in the art is green fluorescent protein (GFP). Fusion domains also include "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion

proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain embodiments, the soluble polypeptides of the present invention contain one or more modifications that are capable of stabilizing the soluble polypeptides. For example, such modifications enhance the in vitro half life of the soluble polypeptides, enhance circulatory half life of the soluble polypeptides or reducing proteolytic degradation of the soluble polypeptides.

In certain embodiments, soluble polypeptides (unmodified or modified) of the invention can be produced by a variety of art-known techniques. For example, such soluble polypeptides can be synthesized using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In addition, automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Bioscience 9600). Alternatively, the soluble polypeptides, fragments or variants thereof may be recombinantly produced using various expression systems as is well known in the art (also see below).

III. Nucleic acids encoding soluble polypeptides

In certain aspects, the invention relates to isolated and/or recombinant nucleic acids encoding an EphB4 or Ephrin B2 soluble polypeptide. The subject nucleic acids may be single-stranded or double-stranded, DNA or RNA molecules. These nucleic acids are useful as therapeutic agents. For example, these nucleic acids are useful in making recombinant soluble polypeptides which are administered to a cell or an individual as therapeutics. Alternatively, these nucleic acids can be directly administered to a cell or an individual as therapeutics such as in gene therapy.

In certain embodiments, the invention provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to a region of the nucleotide sequence depicted in Figure 62 or 63. One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to the subject nucleic acids, and variants of the subject nucleic acids are also within the scope of this invention. In further embodiments, the nucleic acid sequences of the invention can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence depicted in Figure 62 or 63, or complement sequences thereof. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the subject nucleic acids due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In certain embodiments, the recombinant nucleic acids of the invention may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate for a host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences,

leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that
5 combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

10 In certain aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an EphB4 or Ephrin B2 soluble polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the soluble polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other
15 expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a soluble polypeptide. Such useful expression control
20 sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the
25 promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of
30 protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

This invention also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject soluble polypeptide. The host cell may be any prokaryotic or eukaryotic cell. For example, a soluble polypeptide of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject soluble polypeptides. For example, a host cell transfected with an expression vector encoding an EphB4 soluble polypeptide can be cultured under appropriate conditions to allow expression of the EphB4 soluble polypeptide to occur. The EphB4 soluble polypeptide may be secreted and isolated from a mixture of cells and medium containing the soluble polypeptides. Alternatively, the soluble polypeptides may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The soluble polypeptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the soluble polypeptides. In a preferred embodiment, the soluble polypeptide is a fusion protein containing a domain which facilitates its purification.

A recombinant nucleic acid of the invention can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant soluble polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as

pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant SLC5A8 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

IV. Antibodies

In certain aspects, the the present invention provides antagonist antibodies against Ephrin B2 or EphB4. As described herein, the term "antagonist antibody" refers to an antibody that inhibits function of Ephrin B2 or EphB4. Preferably, the antagonist antibody binds to an extracellular domain of Ephrin B2 or EphB4. It is understood that antibodies of

the invention may be polyclonal or monoclonal; intact or truncated, e.g., F(ab')₂, Fab, Fv; xenogeneic, allogeneic, syngeneic, or modified forms thereof, e.g., humanized, chimeric, etc.

For example, by using immunogens derived from an Ephrin B2 or EphB4 polypeptide, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (see, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. (e.g., a polypeptide or an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an Ephrin B2 or EphB4 polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In one embodiment, antibodies of the invention are specific for the extracellular portion of the Ephrin B2 or EphB4 protein. In another embodiment, antibodies of the invention are specific for the intracellular portion or the transmembrane portion of the Ephrin B2 or EphB4 protein. In a further embodiment, antibodies of the invention are specific for the extracellular portion of the Ephrin B2 or EphB4 protein.

Following immunization of an animal with an antigenic preparation of an Ephrin B2 or EphB4 polypeptide, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) *Immunology Today*, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an Ephrin B2 or EphB4 polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with an Ephrin B2 or EphB4 polypeptides. Antibodies can be

fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab)₂ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for an Ephrin B2 or EphB4 polypeptide conferred by at least one CDR region of the antibody. Techniques for the production of single chain antibodies (US Patent No. 4,946,778) can also be adapted to produce single chain antibodies. Also, transgenic mice or other organisms including other mammals, may be used to express humanized antibodies. In preferred embodiments, the antibodies further comprise a label attached thereto and able to be detected (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor).

In certain preferred embodiments, an antibody of the invention is a monoclonal antibody, and in certain embodiments the invention makes available methods for generating novel antibodies. For example, a method for generating a monoclonal antibody that binds specifically to an Ephrin B2 or EphB4 polypeptide may comprise administering to a mouse an amount of an immunogenic composition comprising the Ephrin B2 or EphB4 polypeptide effective to stimulate a detectable immune response, obtaining antibody-producing cells (e.g., cells from the spleen) from the mouse and fusing the antibody-producing cells with myeloma cells to obtain antibody-producing hybridomas, and testing the antibody-producing hybridomas to identify a hybridoma that produces a monoclonal antibody that binds specifically to the Ephrin B2 or EphB4 polypeptide. Once obtained, a hybridoma can be propagated in a cell culture, optionally in culture conditions where the hybridoma-derived cells produce the monoclonal antibody that binds specifically to the Ephrin B2 or EphB4 polypeptide. The monoclonal antibody may be purified from the cell culture.

In addition, the techniques used to screen antibodies in order to identify a desirable antibody may influence the properties of the antibody obtained. For example, an antibody to be used for certain therapeutic purposes will preferably be able to target a particular cell type. Accordingly, to obtain antibodies of this type, it may be desirable to screen for antibodies that bind to cells that express the antigen of interest (e.g., by fluorescence activated cell sorting). Likewise, if an antibody is to be used for binding an antigen in solution, it may be desirable to test solution binding. A variety of different techniques are available for testing antibody:antigen interactions to identify particularly desirable antibodies. Such techniques

include ELISAs, surface plasmon resonance binding assays (e.g. the Biacore binding assay, Bia-core AB, Uppsala, Sweden), sandwich assays (e.g. the paramagnetic bead system of IGEN International, Inc., Gaithersburg, Maryland), western blots, immunoprecipitation assays and immunohistochemistry.

5

V. Drug Screening Assays

There are numerous approaches to screening for polypeptide therapeutic agents as antagonists of EphB4, Ephrin B2 or both. For example, high-throughput screening of compounds or molecules can be carried out to identify agents or drugs which inhibit
10 angiogenesis or inhibit tumor growth. Test agents can be any chemical (element, molecule, compound, drug), made synthetically, made by recombinant techniques or isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and
15 compiled into libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Test agents can also be natural or genetically engineered products isolated from lysates or growth media of cells -- bacterial, animal or plant -- or can be the cell lysates or growth media themselves. Presentation of test compounds to the test system can be in either an isolated
20 form or as mixtures of compounds, especially in initial screening steps.

For example, an assay can be carried out to screen for compounds that specifically inhibit binding of Ephrin B2 (ligand) to EphB4 (receptor), or vice-versa, e.g., by inhibition of binding of labeled ligand- or receptor-Fc fusion proteins to immortalized cells. Compounds identified through this screening can then be tested in animals to assess their anti-
25 angiogenesis or anti-tumor activity in vivo.

In one embodiment of an assay to identify a substance that interferes with interaction of two cell surface molecules (e.g., Ephrin B2 and EphB4), samples of cells expressing one type of cell surface molecule (e.g., EphB4) are contacted with either labeled ligand (e.g., Ephrin B2, or a soluble portion thereof, or a fusion protein such as a fusion of the
30 extracellular domain and the Fc domain of IgG) or labeled ligand plus a test compound (or group of test compounds). The amount of labeled ligand which has bound to the cells is determined. A lesser amount of label (where the label can be, for example, a radioactive

isotope, a fluorescent or colormetric label) in the sample contacted with the test compound(s) is an indication that the test compound(s) interferes with binding. The reciprocal assay using cells expressing a ligand (e.g., an Ephrin B2 ligand or a soluble form thereof) can be used to test for a substance that interferes with the binding of an Eph receptor or soluble portion thereof.

An assay to identify a substance which interferes with interaction between an Eph receptor and an ephrin can be performed with the component (e.g., cells, purified protein, including fusion proteins and portions having binding activity) which is not to be in competition with a test compound, linked to a solid support. The solid support can be any suitable solid phase or matrix, such as a bead, the wall of a plate or other suitable surface (e.g., a well of a microtiter plate), column pore glass (CPG) or a pin that can be submerged into a solution, such as in a well. Linkage of cells or purified protein to the solid support can be either direct or through one or more linker molecules.

In one embodiment, an isolated or purified protein (e.g., an Eph receptor or an ephrin) can be immobilized on a suitable affinity matrix by standard techniques, such as chemical cross-linking, or via an antibody raised against the isolated or purified protein, and bound to a solid support. The matrix can be packed in a column or other suitable container and is contacted with one or more compounds (e.g., a mixture) to be tested under conditions suitable for binding of the compound to the protein. For example, a solution containing compounds can be made to flow through the matrix. The matrix can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds. Compounds which remain bound can be released by a suitable elution buffer. For example, a change in the ionic strength or pH of the elution buffer can lead to a release of compounds. Alternatively, the elution buffer can comprise a release component or components designed to disrupt binding of compounds (e.g., one or more ligands or receptors, as appropriate, or analogs thereof which can disrupt binding or competitively inhibit binding of test compound to the protein).

Fusion proteins comprising all, or a portion of, a protein (e.g., an Eph receptor or an ephrin) linked to a second moiety not occurring in that protein as found in nature can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in which the second moiety comprises an affinity ligand (e.g., an enzyme, antigen, epitope). The fusion proteins can be produced by inserting the protein (e.g., an Eph receptor or an ephrin) or a portion thereof into a suitable expression vector which

encodes an affinity ligand. The expression vector can be introduced into a suitable host cell for expression. Host cells are disrupted and the cell material, containing fusion protein, can be bound to a suitable affinity matrix by contacting the cell material with an affinity matrix under conditions sufficient for binding of the affinity ligand portion of the fusion protein to the affinity matrix.

In one aspect of this embodiment, a fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand portion of the fusion protein to the matrix, and is contacted with one or more compounds (e.g., a mixture) to be tested, under conditions suitable for binding of compounds to the receptor or ligand protein portion of the bound fusion protein. Next, the affinity matrix with bound fusion protein can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds without significantly disrupting binding of specifically bound compounds. Compounds which remain bound can be released by contacting the affinity matrix having fusion protein bound thereto with a suitable elution buffer (a compound elution buffer). In this aspect, compound elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the compound(s) tested to the receptor or ligand protein portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of compounds, or the elution buffer can comprise a release component or components designed to disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein (e.g., one or more ligands or receptors or analogs thereof which can disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein). Immobilization can be performed prior to, simultaneous with, or after contacting the fusion protein with compound, as appropriate. Various permutations of the method are possible, depending upon factors such as the compounds tested, the affinity matrix selected, and elution buffer formulation. For example, after the wash step, fusion protein with compound bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with compound bound thereto. Bound compound can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

VI. Methods of Treatment

In certain embodiments, the present invention provides methods of inhibiting angiogenesis and methods of treating angiogenesis-associated diseases. In other embodiments, the present invention provides methods of inhibiting or reducing tumor growth and methods of treating an individual suffering from cancer. These methods involve administering to the individual a therapeutically effective amount of one or more polypeptide therapeutic agents as described above. These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more particularly, humans.

As described herein, angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; inflammatory disorders such as immune and non-immune inflammation; chronic articular rheumatism and psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation and wound healing; telangiectasia psoriasis scleroderma, pyogenic granuloma, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, hematopoiesis.

It is understood that methods and compositions of the invention are also useful for treating any angiogenesis-independent cancers (tumors). As used herein, the term "angiogenesis-independent cancer" refers to a cancer (tumor) where there is no or little neovascularization in the tumor tissue.

In particular, polypeptide therapeutic agents of the present invention are useful for treating or preventing a cancer (tumor), including, but not limited to, colon carcinoma, breast cancer, mesothelioma, prostate cancer, bladder cancer, squamous cell carcinoma of the head and neck (HNSCC), Kaposi sarcoma, and leukemia.

In certain embodiments of such methods, one or more polypeptide therapeutic agents can be administered, together (simultaneously) or at different times (sequentially). In addition, polypeptide therapeutic agents can be administered with another type of compounds for treating cancer or for inhibiting angiogenesis.

In certain embodiments, the subject methods of the invention can be used alone. Alternatively, the subject methods may be used in combination with other conventional anti-cancer therapeutic approaches directed to treatment or prevention of proliferative disorders (e.g., tumor). For example, such methods can be used in prophylactic cancer prevention, prevention of cancer recurrence and metastases after surgery, and as an adjuvant of other conventional cancer therapy. The present invention recognizes that the effectiveness of conventional cancer therapies (e.g., chemotherapy, radiation therapy, phototherapy, immunotherapy, and surgery) can be enhanced through the use of a subject polypeptide therapeutic agent.

A wide array of conventional compounds have been shown to have anti-neoplastic activities. These compounds have been used as pharmaceutical agents in chemotherapy to shrink solid tumors, prevent metastases and further growth, or decrease the number of malignant cells in leukemic or bone marrow malignancies. Although chemotherapy has been effective in treating various types of malignancies, many anti-neoplastic compounds induce undesirable side effects. It has been shown that when two or more different treatments are combined, the treatments may work synergistically and allow reduction of dosage of each of the treatments, thereby reducing the detrimental side effects exerted by each compound at higher dosages. In other instances, malignancies that are refractory to a treatment may respond to a combination therapy of two or more different treatments.

When a polypeptide therapeutic agent of the present invention is administered in combination with another conventional anti-neoplastic agent, either concomitantly or sequentially, such therapeutic agent is shown to enhance the therapeutic effect of the anti-neoplastic agent or overcome cellular resistance to such anti-neoplastic agent. This allows decrease of dosage of an anti-neoplastic agent, thereby reducing the undesirable side effects, or restores the effectiveness of an anti-neoplastic agent in resistant cells.

Pharmaceutical compounds that may be used for combinatory anti-tumor therapy include, merely to illustrate: aminoglutethimide, amsacrine, anastrozole, asparaginase, bcg, bicalutamide, bleomycin, buserelin, busulfan, camptothecin, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, colchicine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone,

flutamide, gemcitabine, genistein, goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan, ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole, octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, suramin, tamoxifen, temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride, topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and vinorelbine.

These chemotherapeutic anti-tumor compounds may be categorized by their mechanism of action into, for example, following groups: anti-metabolites/anti-cancer agents, such as pyrimidine analogs (5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine) and purine analogs, folate antagonists and related inhibitors (mercaptopurine, thioguanine, pentostatin and 2-chlorodeoxyadenosine (cladribine)); antiproliferative/antimitotic agents including natural products such as vinca alkaloids (vinblastine, vincristine, and vinorelbine), microtubule disruptors such as taxane (paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and navelbine, epididodophyllotoxins (etoposide, teniposide), DNA damaging agents (actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchlorehtamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol, taxotere, teniposide, triethylenethiophosphoramidate and etoposide (VP16)); antibiotics such as dactinomycin (actinomycin D), daunorubicin, doxorubicin (adriamycin), idarubicin, anthracyclines, mitoxantrone, bleomycins, plicamycin (mithramycin) and mitomycin; enzymes (L-asparaginase which systemically metabolizes L-asparagine and deprives cells which do not have the capacity to synthesize their own asparagine); antiplatelet agents; antiproliferative/antimitotic alkylating agents such as nitrogen mustards (mechlorethamine, cyclophosphamide and analogs, melphalan, chlorambucil), ethylenimines and methylmelamines (hexamethylmelamine and thiotepa), alkyl sulfonates-busulfan, nitrosoureas (carmustine (BCNU) and analogs, streptozocin), trazenes - dacarbazine (DTIC); antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones, hormone analogs (estrogen,

tamoxifen, goserelin, bicalutamide, nilutamide) and aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen activator, streptokinase and urokinase), aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; 5 antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus (FK-506), sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF) inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle 10 inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisone, and prednisolone); growth factor signal transduction kinase inhibitors; mitochondrial dysfunction 15 inducers and caspase activators; and chromatin disruptors.

In certain embodiments, pharmaceutical compounds that may be used for combinatory anti-angiogenesis therapy include: (1) inhibitors of release of "angiogenic molecules," such as bFGF (basic fibroblast growth factor); (2) neutralizers of angiogenic molecules, such as an anti- β bFGF antibodies; and (3) inhibitors of endothelial cell response 20 to angiogenic stimuli, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D₃ analogs, alpha-interferon, and the like. For additional proposed inhibitors of angiogenesis, see Blood et al., *Bioch. Biophys. Acta.*, 1032:89-118 (1990), Moses et al., *Science*, 248:1408- 25 1410 (1990), Ingber et al., *Lab. Invest.*, 59:44-51 (1988), and U.S. Pat. Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, and 6,573,256. In addition, there are a wide variety of compounds that can be used to inhibit angiogenesis, for example, peptides or agents that block the VEGF-mediated angiogenesis pathway, endostatin protein or derivatives, lysine binding fragments of angiostatin, melanin or melanin-promoting compounds, plasminogen 30 fragments (e.g., Kringle 1-3 of plasminogen), tropoin subunits, antagonists of vitronectin $\alpha_v\beta_3$, peptides derived from Saposin B, antibiotics or analogs (e.g., tetracycline, or neomycin), dienogest-containing compositions, compounds comprising a MetAP-2 inhibitory core coupled to a peptide, the compound EM-138, chalcone and its analogs, and naaladase

inhibitors. See, for example, U.S. Pat. Nos. 6,395,718, 6,462,075, 6,465,431, 6,475,784, 6,482,802, 6,482,810, 6,500,431, 6,500,924, 6,518,298, 6,521,439, 6,525,019, 6,538,103, 6,544,758, 6,544,947, 6,548,477, 6,559,126, and 6,569,845.

Depending on the nature of the combinatory therapy, administration of the polypeptide therapeutic agents of the invention may be continued while the other therapy is being administered and/or thereafter. Administration of the polypeptide therapeutic agents may be made in a single dose, or in multiple doses. In some instances, administration of the polypeptide therapeutic agents is commenced at least several days prior to the conventional therapy, while in other instances, administration is begun either immediately before or at the time of the administration of the conventional therapy.

VII. Methods of Administration and Pharmaceutical Compositions

In certain embodiments, the subject polypeptide therapeutic agents (e.g., soluble polypeptides or antibodies) of the present invention are formulated with a pharmaceutically acceptable carrier. Such therapeutic agents can be administered alone or as a component of a pharmaceutical formulation (composition). The compounds may be formulated for administration in any convenient way for use in human or veterinary medicine. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Formulations of the subject polypeptide therapeutic agents include those suitable for oral/ nasal, topical, parenteral, rectal, and/or intravaginal administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

In certain embodiments, methods of preparing these formulations or compositions include combining another type of anti-tumor or anti-angiogenesis therapeutic agent and a carrier and, optionally, one or more accessory ingredients. In general, the formulations can

be prepared with a liquid carrier, or a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Formulations for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a subject polypeptide therapeutic agent as an active ingredient.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more polypeptide therapeutic agents of the present invention may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid

esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents
5 such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

In particular, methods of the invention can be administered topically, either to skin or to mucosal membranes such as those on the cervix and vagina. This offers the greatest
10 opportunity for direct delivery to tumor with the lowest chance of inducing side effects. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may
15 further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and sulfur.

Dosage forms for the topical or transdermal administration include powders, sprays,
20 ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The subject polypeptide therapeutic agents may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required. The ointments, pastes, creams and gels may contain, in addition to a subject polypeptide agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins,
25 starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a subject polypeptide therapeutic agent, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain
30 customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Pharmaceutical compositions suitable for parenteral administration may comprise one or more polypeptide therapeutic agents in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Injectable depot forms are made by forming microencapsule matrices of one or more polypeptide therapeutic agents in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

Formulations for intravaginal or rectally administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room

temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

In other embodiments, the polypeptide therapeutic agents of the instant invention can be expressed within cells from eukaryotic promoters. For example, a soluble polypeptide of EphB4 or Ephrin B2 can be expressed in eukaryotic cells from an appropriate vector. The vectors are preferably DNA plasmids or viral vectors. Viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the vectors stably introduced in and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression. Such vectors can be repeatedly administered as necessary. Delivery of vectors encoding the subject polypeptide therapeutic agent can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1. Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins

Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins represent either truncated full-length predicted extracellular domains of Ephrin B2 (B4ECv3, B2EC) or translational fusions of the domains with constant region of human immunoglobulins (IgG1 Fc fragment), such as B2EC-FC, B4ECv2-FC and B4ECv3-FC. Representative human Ephrin B2 constructs and human EphB4 constructs are shown Figures 14 and 15.

The cDNA fragments encoding these recombinant proteins were subcloned into mammalian expression vectors, expressed in transiently or stably transfected mammalian cell lines and purified to homogeneity as described in detail in Materials and Methods section (see

below). Predicted amino acid sequences of the proteins are shown in Figures 1-5. High purity of the isolated proteins and their recognition by the corresponding anti-Ephrin B2 and anti-EphB4 monoclonal or polyclonal antibodies were confirmed. The recombinant proteins exhibit the expected high-affinity binding, binding competition and specificity properties with their corresponding binding partners as corroborated by the biochemical assays (see e.g., Figures 6-8).

Such soluble derivative proteins human Ephrin B2 and EphB4 exhibit potent biological activity in several cell-based assays and *in vivo* assays which measure angiogenesis or anti-cancer activities, and are therefore perspective drug candidates for anti-angiogenic and anti-cancer therapy. B4ECv3 as well as B2EC and B2EC-FC proteins blocked chemotaxis of human endothelial cells (as tested with umbilical cord and hepatic AECs or VECs), with a decrease in degradation of the extracellular matrix, Matrigel, and a decrease in migration in response to growth factor stimuli (Figures 9-11). B4ECv3 and B2EC-FC proteins have potent anti-angiogenic effect as demonstrated by their inhibition of endothelial cell tube formation (Figures 12-13).

Materials and Methods

1) Mammalian expression vectors for producing recombinant soluble derivatives of Ephrin B2 and Eph B4

Plasmids vectors for expressing recombinant soluble derivatives of Ephrin B2 and EphB4 were based on pEF6/V5-His-TOPO vector (Invitrogen), pIG (Novagen) or pRK5. pEF6/V5-His-TOPO contains human elongation factor 1 α enhancer/promoter and blasticidin resistance marker. pIG vector is designed for high-level expression of protein fusions with Fc portion of human IgG1 under CMV promoter control and pRK5 is a general purpose CMV promoter-containing mammalian expression vector. To generate plasmid construct pEF6-B4EC-NT, cDNA fragment of human EphB4 was amplified by PCR using oligo primers 5'-GGATCCGCC ATGGAGCTC CGGGTGCTGCT-3' and 5'-TGGATCCCT GCTCCCGC CAGCCCTCG CTCTCATCCA-3', and TOPO-cloned into pEF6/V5-His-TOPO vector. pEF6-hB4ECv3 was derived from pEF6-B4ECNT by digesting the plasmid DNA with EcoRV and BstBI, filling-in the ends with Klenow enzyme and religating the vector. Recombinant EphB4 derivative encoded by pEF6-B4EC-NT does not contain epitope- or purification tags, while the similar B4ECv3 protein encoded by pEF6-hB4ECv3 contains V5 epitope tag and 6xHis tag on its C-terminus to facilitate purification from conditioned media.

Plasmid construct pEF6-hB2EC was created by PCR amplification of Ephrin B2 cDNA using oligo primers 5'- TGGATCCAC CATGGCTGT GAGAAGGGAC-3' plus 5'- ATTAATGGTGATGGT GAT GATGACTAC CCACTTCGG AACCGAGGATGTTGTTC- 3' and TOPO-cloning into pEF6/V5-His-TOPO vector. Plasmid construct pIG-hB2EC-FC was created by PCR amplification of Ephrin B2 cDNA with oligo primers 5'- TAAAGCTTCCGCCATGG CTGTGAGAAGGGAC-3' and 5'-TAGGATCCACTTCGGA ACCGAGGATGTTGTT CCC-3' , followed by TOPO-cloning and sequencing the resulting PCR fragment with consecutive subcloning in pIG hIgG1 Fc fusion expression vector cut with Bam HI and Hind III. Similarly, pIG-hB2EC and pIG-hB4ECv3 were generated by PCR amplifying portions of EphB4 ECD cDNA using oligo primers 5'-ATAAGCTTCC GCCATGGAGC TCCGGGTGCTG-3' plus 5'-TTGGATCCTGCTCCCG CCAGCCCTCGC TCTCATC-3' with consecutive subcloning into pIG hIgG1 Fc fusion expression vector cut with Bam HI and Hind III. Predicted sequences of the proteins encoded by the vectors described above are shown in Figures 1-5.

2) Mammalian cell culture and transfections

HEK293T (human embryonic kidney line) cells were maintained in DMEM with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. One day before transfections, 293T cells were seeded at a high density to reach 80% confluence at the time of transfection. Plasmid DNA and Lipofectamine reagent at 1:3 ratio were diluted in Opti-MEM I reduced serum medium (Invitrogen) for 5 min and mixed together to form DNA:Lipofectamine complex. For each 10 cm culture dish, 10 µg of plasmid DNA was used. After 20 min, above complex was added directly to cells in culture medium. After 16 hours of transfection, medium was aspirated, washed once with serum free DMEM and replaced with serum free DMEM. Secreted proteins were harvested after 48 hours by collecting conditional medium. Conditional medium was clarified by centrifugation at 10,000 g for 20 min, filtered through 0.2 µm filter and used for purification.

3) Generating stable cell lines

To create stable cell lines producing EphB4ECv3 and EphB4ECnt HEK293 or HEK293T cells were transfected with either pEF6-B4ECv3 or pEF6-B4EC-NT plasmid

constructs as described above and selected using antibiotic Blasticidin. After 24 hours of transfection, cells were seeded at low density. Next day, cells were treated with 10 µg/ml of Blasticidin. After two weeks of drug selection, surviving cells were pooled and selected further for single cell clone expansion. After establishing stable cells, they were maintained at 4 µg/ml Blasticidin. Conditioned media were tested to confirm expression and secretion of the respective recombinant proteins. Specificity of expression was confirmed by Western blot with anti-B4 mono- or polyclonal ABs and B2EC-AP reagent binding and competition assays.

4) Protein purification

HEK293 cells were transiently transfected with a plasmid encoding secreted form of EphB4ectodomain (B4ECv3). Conditional media was harvested and supplemented with 10 mM imidazole, 0.3 M NaCl and centrifuged at 20,000g for 30 min to remove cell debris and insoluble particles. 80 ml of obtained supernatant were applied onto the pre-equilibrated column with 1 ml of Ni-NTA-agarose (Qiagen) at the flow rate of 10 ml/h. After washing the column with 10 ml of 50 mM Tris-HCl, 0.3 M NaCl and 10 mM imidazole, pH 8, remaining proteins were eluted with 3 ml of 0.25 M imidazole. Eluted proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 8 overnight. Purity and identity of B4ECv3 was verified by PAGE/Coomassie G-250 and Western blot with anti-Eph.B4 antibody. Finally, the concentration of B4ECv3 was measured, and the protein was aliquoted and stored at -70 °C.

B4EC-FC protein and B2EC-FC protein were similarly purified.

5) Biochemical Assays

A. binding assay

10 µl of Ni-NTA-Agarose were incubated in microcentrifuge tubes with 50 µl of indicated amount of B4ECv3 diluted in binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin pH 8) After incubation for 30 min on shaking platform, Ni-NTA beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2-AP in the final concentration of 50 nM. Binding was performed for 30 min on shaking platform, and then tubes were centrifuged and washed one time with 1.4 ml of BB. Amount of precipitated AP was measured colorimetrically after application of PNPP.

B. Inhibition assay

Inhibition in solution. Different amounts of B4ECv3 diluted in 50 µl of BB were pre-incubated with 50 µl of 5 nM B2EC-AP reagent (protein fusion of Ephrin B2 ectodomain with placental alkaline phosphatase). After incubation for 1 h, unbound B2EC-AP was
5 precipitated with 5,000 HEK293 cells expressing membrane-associated full-length EphB4 for 20 min. Binding reaction was stopped by dilution with 1.2 ml of BB, followed by centrifugation for 10 min. Supernatants were discarded and alkaline phosphatase activities associated with collected cells were measured by adding para-nitrophenyl phosphate (PNPP) substrate.

10 *Cell based inhibition.* B4ECv3 was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8 and mixed with 5,000 HEK293 cells expressing membrane-associated full-length Ephrin B2. After incubation for 1 h, 50 µl of 5 nM B4EC-AP reagent (protein fusion of EphB4 ectodomain with placental alkaline phosphatase were added into each tube for 30 min to detect unoccupied Ephrin B2 binding sites. Binding reactions were stopped by dilution
15 with 1.2 ml of BB and centrifugation. Colorimetric reaction of cell-precipitated AP was developed with PNPP substrate.

C. B4EC-FC binding assay

Protein A-agarose based assay. 10 µl of Protein A-agarose were incubated in Eppendorf tubes with 50 µl of indicated amount of B4EC-FC diluted in binding buffer BB
20 (20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA pH 8). After incubation for 30 min on shaking platform, Protein A-agarose beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2ECAP reagent at the final concentration of 50 nM. Binding was performed for 30 min on shaking platform, and then tubes were centrifuged and washed once with 1.4 ml of BB. Colorimetric reaction of precipitated AP was measured after application
25 of PNPP (Fig. 6).

Nitrocellulose based assay. B4EC-FC was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 50 µg/ml BSA, pH 8. 2 µl of each fraction were applied onto nitrocellulose strip and spots were dried out for 3 min. Nitrocellulose strip was blocked with 5% non-fat milk for 30 min, followed by incubation with 5 nM B2EC-AP reagent. After 45 min incubation for
30 binding, nitrocellulose was washed twice with 20 mM Tris-HCl, 0.15 M NaCl, 50 µg/ml

BSA, pH 8 and color was developed by application of alkaline phosphatase substrate Sigma Fast (Sigma).

D. B4EC-FC inhibition assay

Inhibition in solution. See above, for B4ECv3. The results were shown in Figure 7.

5 *Cell based inhibition.* See above, for B4ECv3.

E. B2EC-FC binding assay

Protein-A-agarose based assay. See above, for B4EC-FC. The results were shown in Figure 8.

Nitrocellulose based assay. See above, for B4EC-FC.

10 6) Cell-Based Assays

A. Growth Inhibition Assay

Human umbilical cord vein endothelial cells (HUVEC) (1.5×10^3) are plated in a 96-well plate in 100 μ l of EBM-2 (Clonetic # CC3162). After 24 hours (day 0), the test recombinant protein (100 μ l) is added to each well at 2X the desired concentration (5-7
15 concentration levels) in EBM-2 medium. On day 0, one plate is stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates are incubated for 72 h at 37 °C. After 72 h, plates are stained with 0.5% crystal violet in 20% methanol, rinsed with water and airdried. The stain is eluted with 1:1 solution of ethanol: 0.1
20 M sodium citrate (including day 0 plate), and absorbance is measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance is subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC₅₀ (drug concentration causing 50% inhibition) is calculated from the plotted data.

B. Cord Formation Assay (Endothelial Cell Tube Formation Assay)

25 Matrigel (60 μ l of 10 mg/ml; Collaborative Lab # 35423) is placed in each well of an ice-cold 96-well plate. The plate is allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit the matrigel to polymerize. In the mean time, HUVECs are prepared in EGM-2 (Clonetic # CC3162) at a concentration of 2×10^5 cells/ml.

The test compound is prepared at 2X the desired concentration (5 concentration levels) in the same medium. Cells (500 μ l) and 2X drug (500 μ l) is mixed and 200 μ l of this suspension are placed in duplicate on the polymerized matrigel. After 24 h incubation, triplicate pictures are taken for each concentration using a Bioquant Image Analysis system. Drug effect (IC₅₀) is assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

C. Cell Migration Assay

Migration is assessed using the 48-well Boyden chamber and 8 μ m pore size collagen-coated (10 μ g/ml rat tail collagen; Collaborative Laboratories) polycarbonate filters (Osmonics, Inc.). The bottom chamber wells receive 27-29 μ l of DMEM medium alone (baseline) or medium containing chemo-attractant (bFGF, VEGF or Swiss 3T3 cell conditioned medium). The top chambers receive 45 μ l of HUVEC cell suspension (1X10⁶ cells/ml) prepared in DMEM+1% BSA with or without test compound. After 5 h incubation at 37 °C, the membrane is rinsed in PBS, fixed and stained in Diff-Quick solutions. The filter is placed on a glass slide with the migrated cells facing down and cells on top are removed using a Kimwipe. The testing is performed in 4-6 replicates and five fields are counted from each well. Negative unstimulated control values are subtracted from stimulated control and drug treated values and data is plotted as mean migrated cell \pm S.D. IC₅₀ is calculated from the plotted data.

Example 2. Extracellular domain fragments of EphB4 receptor inhibit angiogenesis and tumor growth.

A. Globular domain of EphB4 is required for EphrinB2 binding and for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

To identify subdomain(s) of the ectopic part of EphB4 necessary and sufficient for the anti-angiogenic activity of the soluble recombinant derivatives of the receptor, four recombinant deletion variants of EphB4EC were produced and tested (Fig. 16). Extracellular part of EphB4, similarly to the other members of EphB and EphA receptor family, contains N-terminal ligand-binding globular domain followed by cysteine-rich domain and two fibronectin type III repeats (FNIII). In addition to the recombinant B4-GCF2 protein containing the complete ectopic part of EphB4, we constructed three deletion variants of EphB4EC containing globular domain and Cys-rich domain (B4-GC); globular, Cys-rich and

the first FNIII domain (GCF1) as well as the ECD version with deleted globular domain (CF2). Our attempts to produce several versions of truncated EphB4EC protein containing the globular domain alone were not successful due to the lack of secretion of proteins expressed from all these constructs and absence of ligand binding by the intracellularly expressed recombinant proteins. In addition, a non-tagged version of B4-GCF2, called GCF2-F, containing complete extracellular domain of EphB4 with no additional fused amino acids was expressed, purified and used in some of the experiments described here.

All four C-terminally 6xHis tagged recombinant proteins were preparatively expressed in transiently transfected cultured mammalian cells and affinity purified to homogeneity from the conditioned growth media using chromatography on Ni^{2+} -chelate resin (Fig. 17). Apparently due to their glycosylation, the proteins migrate on SDS-PAAG somewhat higher than suggested by their predicted molecular weights of 34.7 kDa (GC), 41.5 kDa (CF2), 45.6 kDa (GCF1) and 57.8 kDa (GCF2). Sequence of the extracellular domain of human EphB4 contains three predicted N-glycosylation sites (NXS/T) which are located in the Cys-rich domain, within the first fibronectin type III repeat and between the first and the second fibronectin repeats.

To confirm ability of the purified recombinant proteins to bind Ephrin B2, they were tested in an *in vitro* binding assay. As expected, GC, GCF1 and GCF2, but not CF2 are binding the cognate ligand Ephrin B2 as confirmed by interaction between Ephrin B2 – alkaline phosphatase (Ephrin B2-AP) fusion protein with the B4 proteins immobilized on Ni^{2+} -resin or on nitrocellulose membrane (Fig. 17).

All four proteins were also tested for their ability to block ligand-dependent dimerization and activation of Eph B4 receptor kinase in PC3 cells. The PC3 human prostate cancer cell line is known to express elevated levels of human Eph B4. Stimulation of PC3 cells with Ephrin B2 IgG Fc fusion protein leads to a rapid induction of tyrosine phosphorylation of the receptor. However, preincubation of the ligand with GCF2, GCF1 or GC, but not CF2 proteins suppresses subsequent EphB4 autophosphorylation. Addition of the proteins alone to the PC3 cells or preincubation of the cells with the proteins followed by changing media and adding the ligand does not affect EphB4 phosphorylation status.

Further, we found that globular domain of EphB4 is required for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

B. Effects of soluble EphB4 on HUVECs *in vitro*.

Initial experiments were performed to determine whether soluble EphB4 affected the three main stages in the angiogenesis pathway. These were carried out by establishing the effects of soluble EphB4 on migration / invasion, proliferation and tubule formation by HUV/AEC *in vitro*. Exposure to soluble EphB4 significantly inhibited both bFGF and VEGF-induced migration in the Boyden chamber assay in a dose-dependent manner, achieving significance at nM (Fig. 18). Tubule formation by HUV/AECs on wells coated with Matrigel was significantly inhibited by soluble EphB4 in a dose-dependent manner in both the absence and presence of bFGF and VEGF (Fig. 19). We also assessed *in vitro*, whether nM of soluble EphB4 was cytotoxic for HUVECs. Soluble EphB4 was found to have no detectable cytotoxic effect at these doses, as assessed by MTS assay (Fig. 20).

C. Soluble EphB4 receptor Inhibits Vascularization of Matrigel Plugs, *in vivo*

To demonstrate that soluble EphB4 can directly inhibit angiogenesis *in vivo*, we performed a murine matrigel plug experiment. Matrigel supplemented with bFGF and VEGF with and without soluble EphB4 was injected s.c. into Balb/C nu/nu mice, forming semi-solid plugs, for six days. Plugs without growth factors had virtually no vascularization or vessel structures after 6 days (Fig. 21). In contrast, plugs supplemented with bFGF and VEGF had extensive vascularization and vessels throughout the plug. Plugs taken from mice treated with μ g of soluble EphB4 had markedly reduced vascularization of plugs, comparable to plugs without growth factor (Fig. 21). Furthermore, histological examination of plugs showed decreased vessel staining (Fig. 21). Treatment at 0 μ g/dose significantly inhibited the amount of infiltration in Matrigel plugs compared to control (Fig. 21).

We examined EphB4 receptor phosphorylation in HUVECs by performing Western blot analyses with lysates from soluble EphB4-treated cells and antibodies against phosphotyrosine. We found that soluble EphB4 treatment of serum-starved HUVECs stimulated a rapid and transient decrease in the level of phosphorylated EphB4, in the presence of EphrinB2Fc, EphB4 ligand dimer. Ephrin B2Fc without the soluble EphB4 protein induced phosphorylation of EphB4 receptor (Fig. 22).

D. Effects of soluble EphB4 on tumor growth, *in vitro*.

We found that soluble EphB4 inhibits the growth of SCC15 tumors grown in Balb/C Nu/Nu mice (Fig. 23).

E. Soluble EphB4 inhibited corneal neovascularization

To further investigate the antiangiogenic activity of soluble EphB4 *in vivo*, we studied the inhibitory effect of administration of soluble EphB4 on neovascularization in the mouse cornea induced by bFGF. Hydron Pellets implanted into corneal micropocket could induce angiogenesis, in the presence of growth factors, in a typically avascular area. The angiogenesis response in mice cornea was moderate, the appearance of vascular buds was delayed and the new capillaries were sparse and grew slowly. Compared with the control group, on day 7 of implantation, the neovascularization induced by bFGF in mice cornea was markedly inhibited in soluble EphB4-treated group (Fig. 24).

F. Effects of soluble EphB4 on tumor growth, *in vivo*.

The same model was used to determine the effects of soluble EphB4 *in vivo*. SCC15 tumors implanted subcutaneously, pre-incubated with matrigel and with or w/o growth factors, as well as implanted sc alone, and mice treated sc or ip daily with 1-5ug of soluble EphB4 were carried out.

Tumors in the control group continued to grow steadily over the treatment period, reaching a final tumor volume of mm³. However, animals injected with soluble EphB4 exhibited a significantly ($p < 0.05$) reduced growth rate, reaching a final tumor volume of only mm³ (Fig. 25). Similar results were obtained in two further cohorts of such tumor-bearing mice. Soluble EphB4 administration appeared to be well tolerated *in vivo*, with no significant effect on body weight or the general well-being of the animals (as determined by the absence of lethargy, intermittent hunching, tremors or disturbed breathing patterns).

G. Effects of soluble EphB4 on tumor histology.

Histological analysis revealed the presence of a central area of necrosis in all SCC15 tumors, which was usually surrounded by a viable rim of tumor cells um in width. The central necrotic areas were frequently large and confluent and showed loss of cellular detail.

Necrosis, assessed as a percentage of tumor section area, was significantly ($p < 0.02$) more extensive in the soluble EphB4-treated group (% necrosis in treated vs. control). To determine whether the reduced volume of soluble EphB4 treated tumors was due to an effect of this protein on the tumor vascular supply, endothelial cells in blood vessels were identified in tumor sections using immunostaining with an anti-platelet cell adhesion molecule (PECAM-1; CD31) antibody (Fig. 26) and the density of microvessels was assessed. Microvessel density was similar in the outer viable rim of tumor cells (the uniform layer of cells adjacent to the tumor periphery with well defined nuclei) in control and soluble EphB4-

treated tumors. Microvessel density was significantly in the inner, less viable region of tumor cells abutting the necrotic central areas in soluble EphB4-treated than control tumors. Fibrin deposition, as identified by Masson's Trichrome staining, was increased in and around blood vessels in the inner viable rim and the central necrotic core of soluble EphB4 treated than control tumors. In the outer viable rim of soluble EphB4 treated tumors, although the vessel lumen remained patent and contained red blood cells, fibrin deposition was evident around many vessels. Soluble EphB4 was found to have no such effects on the endothelium in the normal tissues examined (lungs, liver and kidneys).

H. Materials and Methods

1) Expression constructs

To construct expression vectors for producing soluble, 6xHis-tagged EphB4-ECD variants, cloned full-length human EphB4 cDNA was amplified by PCR using the following oligo primers: TACTAGTCCGCCATGGAGCTCCGGGTGCTGCT (common EphB4 N-terminal primer) and GCGGCCGCTTAATGGTGATGGTGA TGATGAGCCGAAGGA GGGGTGGTGCA (B4-GC), AGCGGCCGCTTAATGGTGATGGTGAT GATGGACATTGA CAGGCTCAAATGGGA (B4-GCF1) or TGCGGCCGCTTAATGGTGATGGTGATGAT GCTGCTCCCGCCAGCCCTCGCTCTCAT (B4-GCF2). The resulting PCR fragments were TA-cloned into mammalian expression vector pEF6/V5-His-TOPO (Invitrogen) under EF-1 α promoter control. The expressed recombinant proteins encode the following fragments of the mature extracellular part of human EphB4: amino acid positions 1-522 (GCF2), 1-412 (GCF1) and 1-312 (GC). To generate the B4-CF2 deletion (δ amino acids 13-183) PCR fragment for pEF6 cloning, EphB4 cDNA was amplified by two-step overlap PCR using oligo primers TACTAGTCCGCCATGGAGCTCCGGGTGCTGCT, CAGCTGAGTTTCCAATTTTGTGTTC, GAACACAAAATTGGAACTCAGCTGACTGTGAACCTGAC and GCGGCCGCCCCTGCTCCCGCCAGCCCTCGCT.

Vector for producing secreted human EphrinB2-alkaline phosphatase (B2-AP) reagent was constructed by PCR amplification of human Ephrin B2 cDNA using primers TAAAGCTTCCGCCATGGCTGTGAGAAGGGAC and TAGGATCCTTCGGAACCG AGGATGTTGTTCCC and cloning the resulting fragment, digested with Hind III and Bam

HI, into Hind III-Bgl II digested pAPTag2 vector (GenHunter, Inc.). In each case, inserts in expression vectors were verified by complete sequencing.

2) Antibodies and other reagents

Anti-Eph B4 monoclonal antibodies mAB79 and mAB23 were raised in mice against the GCF2 protein containing amino acids 1-522 of mature human EphB4 and purified from hybridoma supernatants by Protein A chromatography. The anti-phosphotyrosine antibody 4G10 was from UBI (Lake Placid, NY). Protein G-HRP conjugate was purchased from Bio-Rad.

3) Expression and purification of EphB4-derived recombinant proteins

To produce the EphB4-ECD soluble proteins, cultured human embryonic kidney cells HEK293T were transfected with the corresponding plasmid constructs using standard calcium phosphate or Lipofectamin 2000 reagent (Invitrogen) protocols. Twelve to sixteen hours post-transfection, the growth medium (DMEM+10% fetal bovine serum) was aspirated, cells washed once with serum free DMEM and replaced with serum free DMEM.

Conditioned media containing the secreted proteins were harvested 72-96 hours later, clarified by centrifugation and used for purification of His-tagged proteins using Ni-NTA Agarose (Qiagen). The purity and quantity of the recombinant proteins was tested by SDS-PAAG electrophoresis with Coomassie Blue or silver staining, Western blotting and UV spectroscopy. Purified proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 8 and stored at -70 °C.

To test ligand binding properties of the proteins, 10 µl of Ni-NTA-Agarose (Qiagen) were incubated in microcentrifuge tubes with 10-500 ng sample of a B4-ECD protein diluted in 0.5 ml of binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin, pH 8). After incubation for 30 min on shaking platform, Ni-NTA beads were washed twice with 1.4 ml of BB, followed by addition of B2-AP fusion protein at concentration of 50 nM. Binding was performed for 30 min on a shaking platform. Tubes were centrifuged and washed once with 1.4 ml of BB. Amount of precipitated AP was measured colorimetrically at 420 nm after application of p-nitrophenyl phosphate (PNPP) and incubation for 5-30 min.

4) Immunoprecipitation

All lysates were processed at 4 °C. Cells were lysed in 1 ml of buffer containing 20 mM Hepes at pH 7.4, 100 mM sodium chloride, 50 mM sodium fluoride, 2 mM EDTA, 2

mM EGTA, 1 mM sodium orthovanadate, 1%(v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM phenyl methylsulphonyl fluoride (added freshly) and 100U Trasylol. Lysates were scraped into Eppendorf tubes and 50 µl of boiled, formalin-fixed *Staphylococcus aureus* was added (Calbiochem, San Diego). After 30 min of mixing, the lysates were centrifuged for 5 min at 25,000g in a minifuge and the supernatants transferred to new tubes containing the appropriate antibody. Lysates were mixed with antibodies for 1 h, after which time 50 µl of protein A-Sepharose beads were added and the contents of the tubes mixed for 1 h to collect the immunoprecipitates. Protein A beads were collected by centrifugation at 25,000g for 30 s. The supernatants were discarded and the beads washed three times with 1 ml lysis buffer minus deoxycholate.

5) Cell-based EphB4 tyrosine kinase assay

The human prostate carcinoma cell line PC3 cells were maintained in RPMI medium with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics mix. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Typically, cells were grown in 60 mm dishes until confluency and were either treated with mouse Ephrin B2-Fc fusion at 1 µg/ml in RPMI for 10 min to activate EphB4 receptor or plain medium as a control. To study the effect of different derivatives of soluble EphB4 ECD proteins on EphB4 receptor activation, three sets of cells were used. In the first set, cells were treated with various proteins (5 proteins; GC, GCF1, GCF2, GCF2-F, CF2) at 5 µg/ml for 20 min. In the second set of cells, prior to application, proteins were premixed with ephrinB2-Fc at 1:5 (EphB4 protein: B2-Fc) molar ratio, incubated for 20 min and applied on cells for 10 min. In the third set of cells, cells were first treated with the proteins for 20 min at 5 µg/ml, media was replaced with fresh media containing 1 µg/ml of EphrinB2-Fc and incubated for another 10 min.

After the stimulation, cells were immediately harvested with protein extraction buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X100, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium vanadate. Protein extracts were clarified by centrifugation at 14,000 rpm for 20 min at 4 °C. Clarified protein samples were incubated overnight with protein A/G coupled agarose beads pre-coated with anti-EphB4 monoclonal antibodies. The IP complexes were washed twice with the same extraction buffer containing 0.1% Triton X100. The immunoprecipitated proteins were solubilized in 1X SDS-PAGE sample loading buffer and separated on 10% SDS-PAGE. For EphB4 receptor activation studies,

electroblotted membrane was probed with anti-pTyr specific antibody 4G10 at 1:1000 dilution followed by Protein G-HRP conjugate at 1:5000 dilutions.

6) Cell Culture

Normal HUVECs were obtained from Cambrex (BioWhittaker) and maintained in EBM2 medium supplemented with 0.1 mg/ml endothelial growth supplement (crude extract from bovine brain), penicillin (50 U/ml), streptomycin (50 U/ml), 2 mmol/l glutamine and 0.1 mg/ml sodium heparin. Aliquots of cells were preserved frozen between passages 1 and 3. For all experiments, HUVECs were used at passages 4 or below and collected from a confluent dish.

7) Endothelial Cell Tube Formation Assay

Matrigel (60 μ l of 10mg/ml; Collaborative Lab, Cat. No. 35423) was placed in each well of an ice-cold 96-well plate. The plate was allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit Matrigel to polymerize. In the mean time, human umbilical vein endothelial cells were prepared in EGM-2 (Clonetic, Cat. No. CC3162) at a concentration of 2×10^5 cells/ml. The test protein was prepared at 2x the desired concentration (5 concentration levels) in the same medium. Cells (500 μ l) and 2x protein (500 μ l) were mixed and 200 μ l of this suspension were placed in duplicate on the polymerized Matrigel. After 24 h incubation, triplicate pictures were taken for each concentration using a Bioquant Image Analysis system. Protein addition effect (IC_{50}) was assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

8) Cell Migration Assay

Chemotaxis of HUVECs to VEGF was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24 well plates, 6.5 mm diam, 8 μ m pore size, 10 μ m thick matrigel coated, polycarbonate membranes (BD Biosciences). The cell suspensions of HUVECs (2×10^5 cells/ml) in 200 μ l of EBM were seeded in the upper chamber and the soluble EphB4 protein were added simultaneously with stimulant (VEGF or bFGF) to the lower compartment of the chamber and their migration across a polycarbonate filter in response to 10- 20 ng/ml of VEGF with or without 100 nM-1 μ M test compound was investigated. After incubation for 4-24 h at 37 °C, the upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Ten random fields at 200x mag were counted and the results expressed as mean # per field. Negative unstimulated control

values were subtracted from stimulated control and protein treated sample values and the data was plotted as mean migrated cell \pm S.D. IC₅₀ was calculated from the plotted data.

9) Growth Inhibition Assay

HUVEC (1.5×10^3 cells) were plated in a 96-well plate in 100 μ l of EBM-2 (Clonetic, Cat. No. CC3162). After 24 hours (day 0), the test recombinant protein (100 μ l) is added to each well at 2x the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 °C. After 72 h, plates were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with 1:1 solution of ethanol: 0.1M sodium citrate (including day 0 plate), and absorbance measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC₅₀ value was calculated from the plotted data.

10) Murine Matrigel Plug Angiogenesis Assay

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/ml, 0.5 ml) in liquid form at 4 °C was mixed with Endothelial Cell Growth Supplement (ECGS), test proteins plus ECGS or Matrigel plus vehicle alone (PBS containing 0.25% BSA). Matrigel (0.5ml) was injected into the abdominal subcutaneous tissue of female nu/nu mice (6 wks old) along the peritoneal mid line. There were 3 mice in each group. The animals were cared for in accordance with institutional and NIH guidelines. At day 6, mice were sacrificed and plugs were recovered and processed for histology. Typically the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin in PBS and embedded in paraffin. Sections of 3 μ m were cut and stained with H&E or Masson's trichrome stain and examined under light microscope

11) Mouse Corneal Micropocket assay

Mouse corneal micropocket assay was performed according to that detailed by Kenyon et al., 1996. Briefly, hydron pellets (polyhydroxyethylmethacrylate [polyHEMA], Interferon Sciences, New Brunswick, NJ, U.S.A.) containing either 90 ng of bFGF (R&D) or 180 ng of VEGF (R&D Systems, Minneapolis, MN, U.S.A.) and 40 μ g of sucrose aluminium

sulfate (Sigma) were prepared. Using an operating microscope, a stromal linear keratotomy was made with a surgical blade (Bard-Parker no. 15) parallel to the insertion of the lateral rectus muscle in an anesthetized animal. An intrastromal micropocket was dissected using a modified von Graefe knife (2~30 mm). A single pellet was implanted and advanced toward the temporal corneal limbus (within $0\pm 7\pm 1\pm 0$ mm for bFGF pellets and 0 ± 5 mm for VEGF pellets). The difference in pellet location for each growth factor was determined to be necessary given the relatively weaker angiogenic stimulation of VEGF in this model. Antibiotic ointment (erythromycin.) was then applied to the operated eye to prevent infection and to decrease surface irregularities. The subsequent vascular response was measured extending from the limbal vasculature toward the pellet and the contiguous circumferential zone of neovascularization. Data and clinical photos presented here were obtained on day 6 after pellet implantation, which was found to be the day of maximal angiogenic response.

12) In vitro invasion assay

"Matrigel" matrix-coated 9-mm cell culture inserts (pore size, 8 μ m; Becton Dickinson, Franklin Lakes, NJ) were set in a 24-well plate. The HUVEC cells were seeded at a density of 5×10^3 cells per well into the upper layer of the culture insert and cultured with serum-free EBM in the presence of EphB4 ECD for 24 h. The control group was cultured in the same media without EphB4. Then 0.5 ml of the human SCC15 cell line, conditioned medium was filled into the lower layer of the culture insert as a chemo-attractant. The cells were incubated for 24 h, then the remaining cells in the upper layer were swabbed with cotton and penetrating cells in the lower layer were fixed with 5% glutaraldehyde and stained with Diff Quick. The total number of cells passing through the Matrigel matrix and each 8 μ m pore of the culture insert was counted using optical microscopy and designated as an invasion index (cell number/area).

13) SCC15 tumor growth in mice

Subcutaneously inject logarithmically growing SCC15, head and neck squamous cell carcinoma cell line, at 5×10^6 cell density; with or without EphB4 ECD in the presence or absence of human bFGF, into athymic Balb/c nude mice, along with Matrigel (BD Bioscience) synthetic basement membrane (1:1 v/v), and examine tumors within 2 weeks. Tumor volumes in the EphB4 ECD group, in the presence and absence of growth factor after implantation were three-fold smaller than those in the vehicle groups. There was no difference in body weight between the groups. Immunohistochemical examination of cross-

sections of resected tumors and TUNEL-positive apoptosis or necrosis, CD34 immunostaining, and BrdU proliferation rate will be performed, after deparaffinized, rehydrated, and quenched for endogenous peroxidase activity, and after 10 min permeabilization with proteinase K. Quantitative assessment of vascular densities will also be performed. Local intratumoral delivery or IV delivery of EphB4 ECD will also be performed twice a week.

30 athymic nude mice, BALB/c (nu/nu), were each injected with 1×10^6 B16 melanoma cells with 0.1 ml PBS mixed with 0.1 ml matrigel or 1.5×10^6 SCC15 cells resuspended in 200 μ l of DMEM serum-free medium and injected subcutaneously on day 0 on the right shoulder region of mice. Proteins were injected intravenously or subcutaneously, around the tumor beginning on day 1 at a loading dose of 4 μ g/mg, with weekly injections of 2 μ g/mg. (10 μ g/g, 50 μ g/kg/day), and at 2 weeks post-inoculation. Mice are sacrificed on Day 14. Control mice received PBS 50 μ l each day.

14) Tumor formation in nude mice

All animals were treated under protocols approved by the institutional animal care committees. Cancer cells (5×10^6) were subcutaneously inoculated into the dorsal skin of nude mice. When the tumor had grown to a size of about 100 mm³ (usually it took 12 days), sEphB4 was either intraperitoneally or subcutaneously injected once/day, and tumorigenesis was monitored for 2 weeks. Tumor volume was calculated according to the formula $a^2 \times b$, where a and b are the smallest and largest diameters, respectively. A Student's t test was used to compare tumor volumes, with $P < .05$ being considered significant.

15) Quantification of microvessel density

Tumors were fixed in 4% formaldehyde, embedded in paraffin, sectioned by 5 μ m, and stained with hematoxylineosin. Vessel density was semi-quantitated using a computer-based image analyzer (five fields per section from three mice in each group).

Example 3. EphB4 Is Upregulated and Imparts Growth Advantage in Prostate Cancer

A. Expression of EphB4 in prostate cancer cell lines

We first examined the expression of EphB4 protein in a variety of prostate cancer cell lines by Western blot. We found that prostate cancer cell lines show marked variation in the abundance of the 120 kD EphB4. The levels were relatively high in PC3 and even higher in

PC3M, a metastatic clone of PC3, while normal prostate gland derived cell lines (MLC) showed low or no expression of EphB4 (Fig. 27A). We next checked the activation status of EphB4 in PC3 cells by phosphorylation study. We found that even under normal culture conditions, EphB4 is phosphorylated though it can be further induced by its ligand, ephrin B2 (Fig. 27B).

B. Expression of EphB4 in clinical prostate cancer samples

To determine whether EphB4 is expressed in clinical prostate samples, tumor tissues and adjacent normal tissue from prostate cancer surgical specimens were examined. The histological distribution of EphB4 in the prostate specimens was determined by immunohistochemistry. Clearly, EphB4 expression is confined to the neoplastic epithelium (Fig. 28, top left), and is absent in stromal and normal prostate epithelium (Fig. 28, top right). In prostate tissue array, 24 of the 32 prostate cancers examined were positive. We found EphB4 mRNA is expressed both in the normal and tumor tissues of clinical samples by quantitative RT-PCR. However, tumor EphB4 mRNA levels were at least 3 times higher than in the normal in this case (Fig. 28, lower right).

C. p53 and PTEN inhibited the expression of EphB4 in PC3 cells

PC3 cells are known to lack PTEN expression (Davis, et al., 1994, Science. 266:816-819) and wild-type p53 function (Gale, et al., 1997, Cell Tissue Res. 290:227-241). We investigated whether the relatively high expression of EphB4 is related to p53 and/or PTEN by re-introducing wild-type p53 and/or PTEN into PC3 cells. To compensate for the transfection efficiency and the dilution effect, transfected cells were sorted for the cotransfected truncated CD4 marker. We found that the expression of EphB4 in PC3 cells was reduced by the re-introduction of either wild-type p53 or PTEN. The co-transfection of p53 and PTEN did not further inhibit the expression of EphB4 (Fig. 29A).

D. Retinoid X receptor (RXR α) regulates the expression of EphB4

We previously found that RXR α was down-regulated in prostate cancer cell lines (Zhong, et al., 2003, Cancer Biol Ther. 2:179-184) and here we found EphB4 expression has the reverse expression pattern when we looked at "normal" prostate (MLC), prostate cancer (PC3), and metastatic prostate cancer (PC3M) (Fig. 27A), we considered whether RXR α regulates the expression of EphB4. To confirm the relationship, the expression of EphB4 was compared between CWR22R and CWR22R-RXR α , which constitutively expresses RXR α . We found a modest decrease in EphB4 expression in the RXR α overexpressing cell line,

while FGF8 has no effect on EphB4 expression. Consistent with initial results, EphB4 was not found in "normal" benign prostate hypertrophic cell line BPH-1 (Fig. 29B).

E. Growth factor signaling pathway of EGFR and IGF-1R regulates EphB4 expression

EGFR and IGF-1R have both been shown to have autocrine and paracrine action on PC3 cell growth. Because we found that EphB4 expression is higher in the more aggressive cell lines, we postulated that EphB4 expression might correlate with these pro-survival growth factors. We tested the relationship by independently blocking EGFR and IGF-1R signaling. EphB4 was down-regulated after blocking the EGFR signaling using EGFR kinase inhibitor AG 1478 (Fig. 30A) or upon blockade of the IGF-1R signaling pathway using IGF-1R neutralizing antibody (Fig. 30B).

F. EphB4 siRNA and antisense ODNs inhibit PC3 cell viability

To define the significance of this EphB4 overexpression in our prostate cancer model, we concentrated our study on PC3 cells, which have a relatively high expression of EphB4. The two approaches to decreasing EphB4 expression were siRNA and AS-ODNs. A number of different phosphorothioate-modified AS-ODNs complementary to different segments of the EphB4 coding region were tested for specificity and efficacy of EphB4 inhibition. Using 293 cells transiently transfected with full-length EphB4 expression vector AS-10 was found to be the most effective (Fig. 31B). A similar approach was applied to the selection of specific siRNA. EphB4 siRNA 472 effectively knocks down EphB4 protein expression (Fig. 31A). Both siRNA 472 and antisense AS-10 ODN reduced the viability of PC3 cells in a dose dependent manner (Fig. 31C, D). Unrelated siRNA or sense oligonucleotide had no effect on viability.

G. EphB4 siRNA and antisense ODNs inhibit the mobility of PC3 Cells

PC3 cells can grow aggressively locally and can form lymph node metastases when injected orthotopically into mice. In an effort to study the role of EphB4 on migration of PC3 cells *in vitro*, we performed a wound-healing assay. When a wound was introduced into a monolayer of PC3 cells, over the course of the next 20 hours cells progressively migrated into the cleared area. However, when cells were transfected with siRNA 472 and the wound was introduced, this migration was significantly inhibited (Fig. 31E). Pretreatment of PC3 cells with 10 μ M EphB4 AS-10 for 12 hours generated the same effect (Fig. 31F). In addition, knock-down of EphB4 expression in PC3 cells with siRNA 472 severely reduced the ability

of these cells to invade Matrigel as assessed by a double-chamber invasion assay (Fig. 31G), compared to the control siRNA.

H. EphB4 siRNA induces cell cycle arrest and apoptosis in PC3 cells

Since knock-down of EphB4 resulted in decreased cell viability (Fig. 31C) we sought to determine whether this was due to effects on the cell cycle. In comparison to control siRNA transfected cells, siRNA 472 resulted in an accumulation of cells in the sub G0 and S phase fractions compared to cells treated with control siRNA. The sub G0 fraction increased from 1 % to 7.9%, and the S phase fraction from 14.9 % to 20.8 % in siRNA 472 treated cells compared to control siRNA treated cells (Fig. 32A). Cell cycle arrest at sub G0 and G2 is indicative of apoptosis. Apoptosis as a result of EphB4 knock-down was confirmed by ELISA assay. A dose-dependent increase in apoptosis was observed when PC3 cells were transfected with siRNA 472, but not with control siRNA (Fig. 32B). At 100 nM there was 15 times more apoptosis in siRNA 472 transfected than control siRNA transfected PC3 cells.

I. Materials and Methods

1) Reagents

Neutralizing IGF-1R antibody was from R&D Systems (Minneapolis MN). Anti-IGF-1R(β), -EGFR, -EphB4(C-16) were from Santa Cruz Biotech (Santa Cruz, CA). β -actin monoclonal antibody was purchased from Sigma Chemical Co. (St Louis, MO). Media and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). AG 1478(4-(3'-Chloroanilino)-6,7-dimethoxy-quinazoline) was from Calbiochem (San Diego, CA).

2) Antisense oligodeoxynucleotides and EphB4 siRNAs

EphB4 specific antisense phosphorothioate-modified oligodeoxynucleotide (ODN) and sense ODN were synthesized and purified by Qiagen (Alameda CA). The sequences are: Sense, 5'-TCC-TGC-AAG-GAG-ACC-TTC-AC-3'; AS1: 5'-GTG-CAG-GGA-TAG-CAG-GGC-CAT-3'; AS10: 5'-ATG-GAG-GCC-TCG-CTC-AGA-AA-3'. siRNAs were synthesized at the USC/Norris Comprehensive Cancer Center Microchemical Core laboratory. Sequences of EphB4 siRNAs are siRNA 472 5'-GGU-GAA-UGU-CAA-GAC-GCU-GUU-3' and siRNA 2303 5'-cuc-uuc-cga-ucc-cac-cua-cuu-3'. Negative control siRNA to scrambled GAPDH was from Ambion (Austin, TX)

3) Cell lines and culture

The prostate cancer cell lines, PC3, PC3M, DU145, ALVA31, LAPC-4, LNCaP, CWR22R and adult human normal prostate epithelial cell line MLC SV40, and BPH-1 were obtained and cultured as described previously (7). Stable cell line CWR22R-RXR, LNCaP-FGF8 were established and cultured as described before (7, 33).

4) Generation of EphB4 monoclonal antibody

The extracellular domain (ECD) of EphB4 was cloned into pGEX-4T-1 to generate GST-fused ECD (GST-ECD). EphB4ECD expressed as a GST fusion protein in BL21 *E. coli* was purified by affinity chromatography and the GST domain was cleaved by thrombin. Monoclonal antibody was generated and the sensitivity and specificity of the antibody was reconfirmed by Western blot with whole cell lysate of 293 cells stably transfected with EphB4.

5) One-Step RT-PCR and Quantitative RT-PCR

Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc. Friendswood TX) from prostate cancer specimens and adjacent normal specimens. For quantitative RT-PCR first strand cDNA was synthesized from 5 µg of total RNA using SuperScript III (Invitrogen, Carlsbad CA). Quantitative RT-PCR was performed on the Stratagene MX3000P system (Stratagene, La Jolla CA) using SYBR Green I Brilliant Mastermix (Stragene) according to the manufacture's instructions. Optimized reactions for EphB4 and β-actin (used as the normalizer gene) were 150 nM each of the forward primer (β-actin, 5'-GGA-CCT-GAC-TGA-CTA-CCT-A-3'; EphB4, 5'-AAG-GAG-ACC-TTC-ACC-GTC-TT-3') and reverse primer (β-actin 5'-TTG-AAG-GTA-GTT-TCG-TGG-AT-3'; EphB4, 5'-TCG-AGT-CAG-GTT-CAC-AGT-CA-3') with DNA denaturation/activation of polymerase at 95 °C for 10 min followed by 40 cycles of 95 °C for 30s, 60 °C for 1min, 72 °C for 1min. The specificity of the gene-specific amplification was confirmed by the presence of a single dissociation peak. All reactions were performed in triplicate with RT and no template negative controls.

6) Immunohistochemistry

OCT-embedded tissues were sectioned at 5 µm and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed for 3 x 5 min in PBS and endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ in PBS for 10 min at room temperature. Sections were incubated with Eph4 (C-16) antibody (1:50) for 1 h at room temperature followed by three washes in PBS and incubation with donkey anti-goat secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. After three washes in PBS, peroxidase activity was

localized by incubation in DAB substrate solution (Vector Laboratories, Inc. Burlingame CA) for 10 min at room temperature. Sections were counterstained with Hematoxylin for 20 s, dehydrated and mounted. Negative control for staining was substitution of normal goat serum for primary antibody. Immunohistochemical staining on prostate array (BioMeda, Foster City, CA) was done using goat ABC Staining System (Santa Cruz Biotech.) according to the manufacturer's instructions.

7) Western blot

Whole cell lysates were prepared using Cell Lysis Buffer (GeneHunter, Basgville TN) supplemented with protease inhibitor cocktail (Pierce, Rockford IL), unless otherwise noted. Total protein was determined using the DC reagent system (Bio-Rad, Hercules CA). Typically, 20 µg whole cell lysate was run on 4-20% Tris-Glycine gradient gel. The samples were electro-transferred to PVDF membrane and the non-specific binding was blocked in TBST buffer (0.5 mM Tris-HCl, 45 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat milk. Membranes were first probed with primary antibody overnight, stripped with RestoreTM Western Blot stripping buffer (Pierce, Rockford IL) and reprobed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

8) Phosphorylation analysis

Cells growing in 60 mm dishes were either serum starved (1% FBS supplemented RPMI 1640, 24 hours) or cultured in normal conditions (10% FBS) and then treated with or without 1 µg/ml mouse ephrin B2/Fc for 10 min to activate EphB4 receptor. Cleared cell lysates were incubated with EphB4 monoclonal antibody overnight at 4 °C. Antigen-antibody complex was immunoprecipitated by the addition of 100 µl of Protein G-Sepharose in 20 mM sodium phosphate, pH 7.0 with incubation overnight at 4 °C. Immunoprecipitates were analyzed by Western blot with pTyr specific antibody (Upstate, clone 4G10) at 1:1000 dilution followed by incubation with protein G-HRP (Bio-Rad) at 1:5000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed with EphB4 specific monoclonal antibody.

9) Transient transfection and sorting of transfected cells

PC3 cells were cotransfected with pMACS 4.1 coding for CD4 and wild type p53 (pC53-SN3) or PTEN vector or both using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The molar ratio of CD4 to p53 or PTEN or vector was 1:3 and

total plasmid was 24 µg for a 10 cm² dish of 90% confluent cells using 60 µl of Lipofectamine 2000. 24 hours after transfection, a single cell suspension was made and sorted using truncated CD4 as a surface marker according to the manufacturer's protocol (Miltenyi Biotec, Germany). Sorted cells were lysed in 1 x SDS sampling buffer and
5 analyzed by Western blot.

10) Study of IGF and EGF signaling pathway on the expression of EphB4

PC3 cells were seeded into 6-well plates and cultured until 80% confluent and treated with 2 µg/ml neutralizing IGF-1R monoclonal antibody, MAB391 (Hailey, et al., 2002, Mol Cancer Ther. 1:1349-1353), or with 1 nM AG 1478, a strong EGFR inhibitor (Liu, et al.,
10 1999, J Cell Sci. 112 (Pt 14):2409-2417) for 24 h. Crude cell lysates were analyzed by Western blot. Band density was quantified with the Bio-Rad QuantityOne System software.

11) Cell viability assay

PC3 cells were seeded on 48-well plates at a density of approximately 1×10^4 cells/well in a total volume of 200 µl. Media was changed after the cells were attached and
15 the cells were treated with various concentrations (1-10 µM) of EphB4 antisense ODN or sense ODN as control. After three days media was changed and fresh ODNs added. Following a further 48 h incubation, cell viability was assessed by MTT as described previously (36). EphB4 siRNAs (10-100 nM) were introduced into 2×10^4 PC3 cells/well of a
48-well plate using 2 µl of Lipofectamine™ 2000 according to the manufacturer's
20 instructions. 4 h post-transfection the cells were returned to growth media (RPMI 1640 supplemented with 10 % FBS). Viability was assayed by MTT 48 h following transfection.

12) Wound healing migration assay

PC3 cells were seeded into 6-well plates and cultured until confluent. 10 µM AS-10 or sense ODN as control were introduced to the wells as described for the viability assay 12
25 hours before wounding the monolayer by scraping it with a sterile pipette tip. Medium was changed to RPMI 1640 supplemented with 5% FBS and fresh ODNs. Confluent cultures transfected with 50 nM siRNA 472 or GAPDH negative control siRNA 12 hours prior to wounding were also examined. The healing process was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera with microscope adapter.

30 13) Invasion assay

PC3 cells were transfected with siRNA 472 or control siRNA using Lipofectamine™ 2000 and 6 hours later 0.5×10^5 cells were transferred into 8 μ m Matrigel-precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing RPMI supplemented with 5 % FBS and 5 μ g/ml fibronectin as a chemoattractant. Following 22 h incubation the inserts were removed and the noninvading cells on the upper surface were removed by with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 min, air dried and stained with Giemsa stain for 2 min. The cells were counted in five individual high-powered fields for each membrane under a light microscope. Assays were performed in triplicate for each treatment group.

14) Cell cycle analysis

80% confluent cultures of PC3 cells in 6-well plates were transfected with siRNA472 (100 nM) using Lipofectamine™ 2000. 24 hours after transfection, cells were trypsinized, washed in PBS and incubated for 1 h at 4°C in 1 ml of hypotonic solution containing 50 μ g/ml propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100 and 20 μ g/ml Dnase-free RnaseA. Cells were analyzed in linear mode at the USC Flow cytometry facility. Results were expressed as percentages of elements detected in the different phases of the cell cycle, namely Sub G0 peak (apoptosis), G0/G1 (no DNA synthesis), S (active DNA synthesis), G2 (premitosis) and M (mitosis).

15) Apoptosis ELISA

Apoptosis was studied using the Cell Death Detection ELISApplus Kit (Roche, Piscataway, NJ) according to the manufacturer's instructions. Briefly, PC3 80% confluent cultures in 24-well plates were transfected using Lipofectamine™ 2000 with various concentrations (0-100 nM) of siRNA 472 or 100 nM control siRNA. 16 hours later, cells were detached and 1×10^4 cells were incubated in 200 μ l lysis buffer. Nuclei were pelleted by centrifugation and 20 μ l of supernatant containing the mono- or oligonucleosomes was taken for ELISA analysis. Briefly, the supernatant was incubated with anti-histone-biotin and anti-DNA-POD in streptavidin-coated 96-well plate for 2 hours at room temperature. The color was developed with ABST and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA).

Example 4. Expression of EPHB4 in Mesothelioma: a candidate target for therapy

Malignant mesothelioma (MM) is a rare neoplasm that most often arises from the pleural and peritoneal cavity serous surface. The pleural cavity is by far the most frequent site affected (> 90%), followed by the peritoneum (6-10%) (Carbone et al., 2002, Semin Oncol. 29:2-17). There is a strong association with asbestos exposure, about 80% of malignant mesothelioma cases occur in individuals who have ingested or inhaled asbestos. This tumor is particularly resistant to the current therapies and, up to now, the prognosis of these patients is dramatically poor (Lee et al., 2000, Curr Opin Pulm Med. 6:267-74).

Several clinical problems regarding the diagnosis and treatment of malignant mesothelioma remain unsolved. Making a diagnosis of mesothelioma from pleural or abdominal fluid is notoriously difficult and often requires a thoracoscopic or laproscopic or open biopsy and Immunohistochemical staining for certain markers such as mesothelin expressed preferentially in this tumor. Until now, no intervention has proven to be curative, despite aggressive chemotherapeutic regimens and prolonged radiotherapy. The median survival in most cases is only 12-18 months after diagnosis.

In order to identify new diagnostic markers and targets to be used for novel diagnostic and therapeutic approaches, we assessed the expression of EPHB4 and its ligand EphrinB2 in mesothelioma cell lines and clinical samples.

A. EPHB4 and EphrinB2 is expressed in mesothelioma cell lines

The expression of Ephrin B2 and EphB4 in malignant mesothelioma cell lines was determined at the RNA and protein level by a variety of methods. RT-PCR showed that all of the four cell lines express EphrinB2 and EPHB4 (fig. 33A). Protein expression was determined by Western blot in these cell lines. Specific bands for EphB4 were seen at 120 kD. In addition, Ephrin B2 was detected in all cell lines tested as a 37 kD band on Western blot (fig. 33B). No specific band for Ephrin B2 was observed in 293 human embryonic kidney cells, which were included as a negative control.

To confirm the presence of EphB4 transcription in mesothelioma cells, *in situ* hybridization was carried out on NCI H28 cell lines cultured on chamber slides. Specific signal for EphB4 was detected using antisense probe Ephrin B2 transcripts were also detected in the same cell line. Sense probes for both EphB4 and Ephrin B2 served as negative controls and did not hybridize to the cells (figure 34). Expression of EphB4 and Ephrin B2 proteins was confirmed in the cell lines by immunofluorescence analysis (fig. 35). Three cell lines

showed strong expression of EphB4, whereas expression of Ephrin B2 was present in H28 and H2052, and weakly detectable in H2373.

B. Evidence of Expression of EPHB4 and EphrinB2 in clinical samples

Tumor cells cultured from the pleural effusion of a patient diagnosed with pleural malignant mesothelioma were isolated and showed positive staining for both EphB4 and Ephrin B2 at passage 1 (figure 35, bottom row). These results confirm co-expression of EphB4 and Ephrin B2 in mesothelioma cell lines. To determine whether these results seen in tumor cell lines were a real reflection of expression in the disease state, tumor biopsy samples were subjected to immunohistochemical staining for EphB4 and Ephrin B2. Antibodies to both proteins revealed positive stain in the tumor cells. Representative data is shown in figure 36.

C. EPHB4 is involved in the cell growth and migration of mesothelioma

The role of EphB4 in cell proliferation was tested using EPHB4 specific antisense oligonucleotides and siRNA. The treatment of cultured H28 with EPHB4 antisense reduced cell viability. One of the most active inhibitor of EphB4 expression is EPHB4AS-10 (fig. 37A). Transfection of EPHB4 siRNA 472 generated the same effect (fig. 37B).

MM is a locally advancing disease with frequent extension and growth into adjacent vital structures such as the chest wall, heart, and esophagus. In an effort to study this process in vitro, we perform wound healing assay using previously described techniques (3:36). When a wound was introduced into sub confluent H28 cells, over the course of the next 28 hours cells would progressively migrate into the area of the wound. However, when cells were pretreated with EPHB4AS-10 for 24 hours, and the wound was introduced, this migration was virtually completely prevented (fig. 38A). The migration study with Boyden Chamber assay with EPHB4 siRNA showed that cell migration was greatly inhibited with the inhibition of EPHB4 expression (Fig. 38B).

D. Materials and Methods

1) Cell lines and reagents

NCI H28, NCI H2052, NCI H2373, MSTO 211H mesothelioma cell lines and 293 human embryonic kidney cells were obtained from the ATCC (Manassas, VA). Cells were maintained in RPMI 1640 media supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and antibiotics. Primary cells were obtained

from pleural effusion of patients with mesothelioma. A large number of EPHB4 phosphorothioate modified antisense oligonucleotides were synthesized. Similarly a number of EphB4 specific siRNAs were generated. Monoclonal antibody produced against EPHB4 was used for western blot. Polyclonal antibody against EphrinB2 and EPHB4 (C-16) (for immunohistochemical staining) was from Santa Cruz.

2) RT-PCR

Total RNA was reversed transcribed by use of random hexamers (Invitrogen). Primers for EphB4 and EphrinB2 were designed with Primer 3 software. The sequences for all primers are as follows: EPHB4 forward primer and EPHB4 reverse primer (see, e.g., in Example 2); EphrinB2 forward primer and EphrinB2 reverse primer (see, e.g., in Example 6); G3PDH forward primer, 5'-GGAGCCAAAAGGGTCATCAT-3'; G3PDH reverse primer, 5'-GGCATTGCTGCAAAGAAAGAG-3'; Clonetics kit was used for PCR. PCRs were performed with the ABI PCR System 2700 (Applied Biosystem). The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1min.

3) Preparation of digoxigenin-labeled RNA probes

Ephrin-B2 and EphB4 PCR products were cloned using the pGEM-T Easy System (Promega, Madison WI) according to the manufacturer's description. The primers and PCR products were 5'-tccgtgtggaagtactgtg-3' (forward), 5'-tctggttgccacagttgag-3' (reverse), for ephrin-B2 that yielded a 296-bp product and 5'-ctttggaagagaccctgtg-3' (forward), 5'-agacgggtgaaggtctccttg-3', for EphB4 that yielded a 297-bp product. The authenticity and insert orientation were confirmed by DNA sequencing.

The pGEM-T Easy plasmids containing the PCR product of the human ephrin-B2 or EphB4 gene were linearized with *Spe* I or *Nco* I. Antisense or sense digoxigenin (DIG)-labeled RNA probes were transcribed from T7 or SP6 promoters by run-off transcription using a DIG RNA labeling kit (Roche, Indianapolis IN). RNA probes were quantitated by spot assay as described in the DIG RNA labeling kit instructions.

4) *In situ* hybridization

Cells were cultured in Labtech II 4-well chamber slides (Nalge Nunc International, Naperville, IL). Cells were washed in PBS (37 °C), then fixed for 30 min at 25 °C in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl. After fixation, slides were rinsed with PBS and stored in 70% ethanol at 4 °C until further use.

Before *in situ* hybridization, cells were dehydrated, washed in 100% xylene to remove residual lipid and then rehydrated, finally in PBS. Cells were permeabilized by incubating at 37 °C with 0.1% (w/v) pepsin in 0.1 N HCl for 20 min and post-fixed in 1% formaldehyde for 10 min. Prehybridization was performed for 30 min at 37 °C in a solution of 4 X SSC containing 50%(v/v) deionized formamide. Slides were hybridized overnight at 42 °C with 25 ng antisense or sense RNA probes in 40% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 4 X SSC, 10 mM DTT, 1 mg/ml yeast t-RNA and 1mg/ml denatured and sheared salmon sperm DNA in a total volume of 40 µl. Slides were then washed at 37 °C as follows: 2 X 15 min with 2 X SSC, 2 X 15min with 1 X SSC, 2 X 15 min with 0.5 X SSC and 2 X 30 min with 0.2 X SSC. Hybridization signal was detected using alkaline-phosphatase-conjugated anti-DIG antibodies (Roche) according to the manufacturer's instructions. Color development was stopped by two washes in 0.1 M Tris-HCl, 1mM EDTA, pH 8.0 for 10 min. Cells were visualized by counterstaining of nucleic acids with Nuclear Fast Red (Vector Laboratories, Burlingame, CA) and the slides were mounted with IMMUMOUNT (Shandon, Astmoor UK).

5) Western Blot

Crude cell lysates were prepared by incubation in cell lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 1 mM DTT, 10 % glycerol). Lysates were cleared by centrifugation at 10,000 x g for 10 min. Total protein was determined by Bradford assay (Bio-Rad). Samples (20 µg protein) were fractionated on a 4-20 % Tris-glycine polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by electroblotting. Membranes were blocked with 5 % non-fat milk prior to incubation with antibody to EphB4 (1:5000 dilution) at 4° C, for 16 h. Secondary antibody (1:100,000 dilution) conjugated with horseradish peroxidase was applied for 1 h at 25 °C. The membranes were developed using the SuperSignal West Femto Maximum sensitivity chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

6) Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized and incubated with 10% goat serum at -70 °C for 10 minutes and incubated with the primary rabbit antibodies against either Ephrin B2 or EphB4 (Santa Cruz Biotechnologies; 1:100) at 4 °C overnight. Isotype-specific rabbit IgG was used as control. The immunoreactivity for these receptors was

revealed using an avidin-biotin kit from Vector Laboratories. Peroxidase activity was revealed by the diaminobenzidine (Sigma) cytochemical reaction. The slides were then counterstained with H&E.

7) Immunofluorescence studies

5 Cells were cultured on Labtech II 4-well chamber slides and fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline pH 7.4 (PBS) for 30 min. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton-X100, 1% BSA in PBS) for 20 min. The slides were then incubated with antibodies to EphB4 or ephrin B2 (1:100 dilution in PBS) in blocking buffer at 4 °C for 16 hr. After washing three
10 times, the slides were incubated with the appropriate fluorescein-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), washed extensively with PBS and mounted with Vectasheild antifade mounting solution (Vector Laboratories). Images were obtained using an Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments
15 Inc., Sterling Heights, MI) digital imaging system.

8) Cell viability assay

Cells were seeded at a density of 5×10^3 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). On the following day, the media was changed and cells were treated with various concentrations (1-10 μ M) of EphB4
20 Antisense. On day 4, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml. Cells were incubated for 2 hr, medium was aspirated, and the cells were dissolved in acidic isopropanol (90% isopropanol, 0.5% SDS and 40 mM HCl). Optical density was read in an ELISA reader at 490 nm using isopropanol as blank (Molecular Devices, CA).

25 9) Cell migration

In vitro wound healing assay was adopted. Briefly, cells were seeded onto 6-cm plates in full culture media for 24 hours, and then switched to medium containing 5% FBS. EPHB4 antisense 10 (10 μ M) was also added to treated well. 24 hours later, wounds were made using the tip of a p-200 pipette man; a line was drawn through the middle of the plates. The plate
30 was photographed at 0, 12, 24 hours. The experiment was repeated three times.

Example 5. EphB4 Is Expressed in Squamous Cell Carcinoma of The Head and Neck:
Regulation by Epidermal Growth Factor Signaling Pathway and Growth Advantage.

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide, with estimated 900,000 cases diagnosed each year. It comprises almost 50% of all malignancies in some developing nations. In the United States, 50,000 new cases and 8,000 deaths are reported each year. Tobacco carcinogens are believed to be the primary etiologic agents of the disease, with alcohol consumption, age, gender, and ethnic background as contributing factors.

The differences between normal epithelium of the upper aerodigestive tract and cancer cells arising from that tissue are the result of mutations in specific genes and alteration of their expression. These genes control DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis. For head and neck cancer, alterations of three signaling pathways occur with sufficient frequency and produce such dramatic phenotypic changes as to be considered the critical transforming events of the disease. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC.

Although high expression of EphB4 has been reported in hematologic malignancies, breast carcinoma, endometrial carcinoma, and colon carcinoma, there is limited data on the protein levels of EphB4, and complete lack of data on the biological significance of this protein in tumor biology such as HNSCC.

A. HNSCC tumors express EphB4

We studied the expression of EphB4 in human tumor tissues by immunohistochemistry, in situ hybridization, and Western blot. Twenty prospectively collected tumor tissues following IRB approval have been evaluated with specific EphB4 monoclonal antibody that does not react with other members of the EphB and EphA family. EphB4 expression is observed in all cases, with varying intensity of staining. Figure 39A (top left) illustrates a representative case, showing that EphB4 is expressed in the tumor regions only, as revealed by the H&E tumor architecture (Fig. 39A bottom left). Note the absence of

staining for EphB4 in the stroma. Secondly, a metastatic tumor site in the lymph node shows positive staining while the remainder of the lymph node is negative (Fig. 39A, top right).

In situ hybridization was carried out to determine the presence and location of EphB4 transcripts in the tumor tissue. Strong signal for EphB4 specific antisense probe was detected indicating the presence of transcripts (Figure 39 B, top left). Comparison with the H&E stain (Fig. 39B, bottom left) to illustrate tumor architecture reveals that the signal was localized to the tumor cells, and was absent from the stromal areas. Ephrin B2 transcripts were also detected in tumor sample, and as with EphB4, the signal was localized to the tumor cells (Fig. 39B, top right). Neither EphB4 nor ephrin B2 sense probes hybridized to the sections, proving specificity of the signals.

B. High expression of EphB4 in primary and metastatic sites of HNSCC

Western blots of tissue from primary tumor, lymph node metastases and uninvolved tissue were carried out to determine the relative levels of EphB4 expression in these sites. Tumor and normal adjacent tissues were collected on 20 cases, while lymph nodes positive for tumor were harvested in 9 of these 20 cases. Representative cases are shown in figure 39C. EphB4 expression is observed in each of the tumor samples. Similarly, all tumor positive lymph nodes show EphB4 expression that was equal to or greater than the primary tumor. No or minimal expression is observed in the normal adjacent tissue.

C. EphB4 expression and regulation by EGFR activity in HNSCC cell lines

Having demonstrated the expression of EphB4 limited to tumor cells, we next sought to determine whether there was an in vitro model of EphB4 expression in HNSCC. Six HNSCC cell lines were surveyed for EphB4 protein expression by Western Blot (Fig. 40A). A majority of these showed strong EphB4 expression and thus established the basis for subsequent studies. Since EGFR is strongly implicated in HNSCC we asked whether EphB4 expression is associated with the activation of EGFR. Pilot experiments in SCC-15, which is an EGFR positive cell line, established an optimal time of 24 h and concentration of 1 mM of the specific EGFR kinase inhibitor AG 1478 (Figure 40B) to inhibit expression of EphB4. When all the cell lines were studied, we noted robust EGFR expression in all but SCC-4, where it is detectable but not strong (Fig. 40C, top row). In response to EGFR inhibitor AG1478 marked loss in the total amount of EphB4 was observed in certain cell lines (SCC-15, and SCC-25) while no effect was observed in others (SCC-9, -12, -13 and -71). Thus SCC-15 and -25 serve as models for EphB4 being regulated by EGFR activity, while SCC-9,

-12, -13 and -71 are models for regulation of EphB4 in HNSCC independent of EGFR activity, where there may be input from other factors such as p53, PTEN, IL-6 etc. We also noted expression of the ligand of EphB4, namely ephrin B2, in all of the cell lines tested. As with EphB4 in some lines ephrin B2 expression appears regulated by EGFR activity, while it is independent in other cell lines.

Clearly, inhibition of constitutive EGFR signaling repressed EphB4 levels in SCC15 cells. We next studied whether EGF could induce EphB4. We found that EphB4 levels were induced in SCC15 cells that had been serum starved for 24 h prior to 24 h treatment with 10 ng/ml EGF as shown in figure 41B (lanes 1 and 2). The downstream signaling pathways known for EGFR activation shown in figure 41A, (for review see Yarden & Slikowski 2001) were then investigated for their input into EGF mediated induction of EphB4. Blocking PLCg, AKT and JNK phosphorylation with the specific kinase inhibitors U73122, SH-5 and SP600125 respectively reduced basal levels and blocked EGF stimulated induction of EphB4 (Fig. 41B, lanes 3-8). In contrast, inhibition of ERK1/2 with PD098095 and PI3-K with LY294002 or Wortmannin had no discernible effect on EGF induction of EphB4 levels. However, basal levels of EphB4 were reduced when ERK1/2 phosphorylation was inhibited. Interestingly, inhibition of p38 MAPK activation with SB203580 increased basal, but not EGF induced EphB4 levels. Similar results were seen in the SCC25 cell line (data not shown).

D. Inhibition of EphB4 in high expressing cell lines results in reduced viability and causes cell-cycle arrest

We next turned to the role of EphB4 expression in HNSCC by investigating the effect of ablating expression using siRNA or AS-ODN methods. Several siRNAs to EphB4 sequence were developed (Table 1) which knocked-down EphB4 expression to varying degrees as seen in figure 42A. Viability was reduced in SCC-15, -25 and -71 cell lines transfected with siRNAs 50 and 472, which were most effective in blocking EphB4 expression (Figure 42B). Little effect on viability was seen with EphB4 siRNA 1562 and 2302 or ephrin B2 siRNA 254. Note that in SCC-4, which does not express EphB4 (see Fig. 40A) there was no reduction in cell viability. The decreased cell viability seen with siRNA 50 and 472 treatment was attributable to accumulation of cells in sub G0, indicative of apoptosis. This effect was both time and dose-dependant (Figure 42C and Table 2). In contrast, siRNA2302 that was not effective in reducing EphB4 levels and had only minor effects on

viability did not produce any changes in the cell cycle when compared with the mock LipofectamineTM2000 transfection.

Table 1: EphB4 siRNAs

Name	siRNA sequence
Eph B4 50:	5' - GAGACCCUGCUGAACACAAUU - 3' 3' - UUCUCUGGGACGACUUGUGUU - 5'
Eph B4 472:	5' - GGUGAAUGUCAAGACGCUGUU - 3' 3' - UUCCACUUACAGUUCUGCGAC - 5'
Eph B4 1562:	5' - CAUCACAGCCAGACCCAACUU - 3' 3' - UUGUAGUGUCGGUCUGGGUUG - 5'
Eph B4 2302	5' - CUCUCCGAUCCACCUACUU - 3' 3' - UUGAGAAGGCUAGGGUGGAUG - 5'

5 Table 2: Effect of different EphB4 siRNA on Cell Cycle

Treatment	Sub G0	G1	S	G2
36hr				
Lipo alone	1.9	39.7	21.3	31.8
100 nM 2302	2.0	39.3	21.2	31.2
100 nM 50	18.1	31.7	19.7	24.4
100 nM 472	80.2	10.9	5.2	2.1
16hr				
Lipo alone	7.8	55.7	15.2	18.5
100 nM 2302	8.4	57.3	14.3	17.3
10 nM 50	10.4	53.2	15.7	17.7
100 nM 50	27.7	31.3	18.1	19.6
10 nM 472	13.3	50.2	15.8	17.5
100 nM 472	30.7	31.9	16.4	18.0

In addition, over 50 phosphorothioate AS-ODNs complementary to the human EphB4 coding sequences were synthesized and tested for their ability to inhibit EphB4 expression in 293 cells transiently transfected with full length EphB4 expression plasmid. Figure 43A shows a representative sample of the effect of some of these AS-ODNs on EphB4 expression.

5 Note that expression is totally abrogated with AS-10, while AS-11 has only a minor effect. The effect on cell viability in SCC15 cells was most marked with AS-ODNs that are most effective in inhibiting EphB4 expression as shown in figure 43B. The IC₅₀ for AS-10 was approximately 1 μ M, while even 10 μ M AS-11 was not sufficient to attain 50 % reduction of viability. When the effect that AS-10 had on the cell cycle was investigated, it was found that
10 the sub G0 fraction increased from 1.9 % to 10.5 % compared to non-treated cells, indicative of apoptosis (Fig. 43C).

E. EphB4 regulates Cell migration

We next wished to determine if EphB4 participates in the migration of HNSCC. Involvement in migration may have implications for growth and metastasis. Migration was
15 assessed using the wound-healing/scrape assay. Confluent SCC15 and SCC25 cultures were wounded by a single scrape with a sterile plastic Pasteur pipette, which left a 3 mm band with clearly defined borders. Migration of cells into the cleared area in the presence of test compounds was evaluated and quantitated after 24, 48 and 72 hr. Cell migration was markedly diminished in response to AS-10 that block EphB4 expression while the inactive
20 compounds, AS-1 and scrambled ODN had little to no effect as shown in figure 43D. Inhibition of migration with AS-10 was also shown using the Boyden double chamber assay (Fig. 43E).

F. EphB4 AS-10 in vivo anti-tumor activity

The effect of EphB4 AS-10, which reduces cell viability and motility, was determined
25 in SCC15 tumor xenografts in Balb/C nude mice. Daily treatment of mice with 20 mg/kg AS-10, sense ODN or equal volume of PBS by I.P. injection was started the day following tumor cell implantation. Growth of tumors in mice receiving AS-10 was significantly retarded compared to mice receiving either sense ODN or PBS diluent alone (Figure 44). Non-specific effects attributable to ODN were not observed, as there was no difference between the sense
30 ODN treated and PBS treated groups.

G. Materials and Methods

1) Cell lines and reagents

HNSCC-4, -9, 12, -13, -15, -25, and -71 were obtained from and 293 human embryonic kidney cells were obtained from the ATCC (Manassas, VA). Cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and antibiotics. EGFR, EphB4(C-16) polyclonal antibodies were from Santa Cruz Biotech (Santa Cruz, CA). β -actin monoclonal antibody was purchased from Sigma Chemical Co. (St Louis, MO). Ephrin B2 and EphB4 polyclonal antibodies and their corresponding blocking peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AG 1478 (4-(3'-Chloroanilino)-6,7-dimethoxy-quinazoline) was from Calbiochem (San Diego, CA). Kinase inhibitors SH-5 and SP 600125 were from A.G. Scientific (San Diego, CA), PD98095, U73122, SB203580, LY294002, and Wortmannin were obtained from Sigma.

2) Preparation of digoxigenin-labeled RNA probes

See above, e.g., Example 3.

3) *In situ* hybridization

See above, e.g., Example 3.

4) Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized and incubated with 10% goat serum at -70 °C for 10 minutes and incubated with the EphB4 monoclonal antibody 4 °C overnight. Isotype specific rabbit IgG was used as control. The immunoreactivity for these receptors was revealed using an avidin-biotin kit from Vector Laboratories. Peroxidase activity was revealed by the diaminobenzidine (Sigma) cytochemical reaction. The slides were then counterstained with 0.12% methylene blue or H&E. For frozen sections, OCT-embedded tissues were sectioned at 5 μ m and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed for 3 x 5 min in PBS and endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ in PBS for 10 min at room temperature. Sections were incubated with Eph4 (C-16) antibody (1:50) for 1 h at room temperature followed by three washes in PBS and incubation with donkey anti-goat secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. After three washes in PBS, peroxidase activity was localized by incubation in DAB substrate solution (Vector Laboratories, Inc. Burlingame CA) for 10 min at room temperature. Sections were counterstained with Hematoxylin for 20 s, dehydrated and mounted. Negative control for staining was substitution of normal goat serum for primary antibody. Immunohistochemical staining on prostate array (BioMeda, Foster

City, CA) was done using goat ABC Staining System (Santa Cruz Biotech.) according to the manufacturer's instructions.

5) Western Blot

See above, e.g., Example 3.

5 6) Synthesis of EphB4 siRNA by in vitro transcription

The SilencerTM siRNA construction kit (Ambion, Austin TX) was used to synthesize siRNA to EphB4. Briefly, 21 bp target sequences containing 19 bp downstream of 5'-AA dinucleotides were identified that showed no significant homology to other sequences in the GenBank database. Sense and antisense siRNA 29-mer DNA oligonucleotide templates were synthesized at the USC Norris Microchemical Core Facility. Antisense template corresponded to the target sequence followed by 8 bp addition (5'-CCTGTCTC-3') at the 3' end complementary to the T7 promoter primer provided by the SilencerTM siRNA construction kit. Sense template comprised 5'-AA followed by the complement of the target 19 bp, then the T7 8 bp sequence as above.

15 In separate reactions, the two siRNA oligonucleotide templates were hybridized to a T7 promoter primer. The 3' ends of the hybridized oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The leader sequences were removed by digesting the dsRNA with a single-stranded specific ribonuclease leaving the overhanging UU dinucleotides. The DNA template was removed at the same time by treatment with RNase free deoxyribonuclease. The resulting siRNA was purified by glass fiber filter binding to remove excess nucleotides, short oligomers, proteins, and salts in the reaction. The end products (shown in Table 3) were double-stranded 21-mer siRNAs with 3' terminal uridine that can effectively reduce the expression of target mRNA when transfected into cells.

A number of phosphorothioate AS-ODNs were also synthesized (Operon, Valencia CA) to test for inhibition of EphB4 expression (Table 3).

Table 3: EphB4 Antisense ODNs

Name	Position	Sequence (5' → 3')
------	----------	--------------------

Eph B4 AS-1	(552-572)	GTG CAG GGA TAG CAG GGC CAT
Eph B4 AS-2	(952-972)	AAG GAG GGG TGG TGC ACG GTG
Eph B4 AS-3	(1007-1027)	TTC CAG GTG CAG GGA GGA GCC
Eph B4 AS-4	(1263-1285)	GTG GTG ACA TTG ACA GGC TCA
Eph B4 AS-5	(1555-1575)	TCT GGC TGT GAT GTT CCT GGC
Eph B4 AS-6	(123-140)	GCC GCT CAG TTC CTC CCA
Eph B4 AS-7	(316-333)	TGA AGG TCT CCT TGC AGG
Eph B4 AS-8	(408-428)	CGC GGC CAC CGT GTC CAC CTT
Eph B4 AS-9	(1929-1949)	CTT CAG GGT CTT GAT TGC CAC
Eph B4 AS-10	(1980-1999)	ATG GAG GCC TCG CTC AGA AA
Eph b4 AS-11	(2138-2158)	CAT GCC CAC GAG CTG GAT GAC

7) Cell viability assay

Cells were seeded at a density of 5×10^3 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). Cells were treated with various concentrations (1-10 $\mu\text{g/ml}$) of ODNs on days 2 and 4. On day 5, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (Masood et al '03). For viability with siRNA, 2×10^4 cells/well of SCC-4, -15, -25 or -71 in a 48-well plate were transfected with siRNAs (10-100 nM) using 2 μl of Lipofectamine™ 2000 according to the manufacturer's instructions. 4 h post-transfection the cells were returned to growth media (RPMI 1640 supplemented with 10 % FBS). Viability was assayed by MTT 48 h following transfection.

8) Cell cycle analysis

80% confluent cultures of SCC15 cells in 6-well plates were transfected with siRNA472 (100 nM) using Lipofectamine™ 2000. Either 16 or 36 hours after transfection, cells were trypsinized, washed in PBS and incubated for 1 h at 4 °C in 1 ml of hypotonic solution containing 50 $\mu\text{g/ml}$ propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100 and 20 $\mu\text{g/ml}$ DNase-free RNaseA. Cells were analyzed in linear mode at the USC Flow cytometry facility. Results were expressed as percentages of elements detected in the different phases of the cell cycle, namely Sub G0 peak (apoptosis), G0/G1 (no DNA synthesis), S (active DNA

synthesis), G2 (premitosis) and M (mitosis). For AS-ODN experiment the cells were exposed to 5 μ M ODN for 36 h prior to processing.

9) Wound healing migration assay

SCC15 cells were seeded into 6-well plates and cultured until confluent. 10 μ M AS-1, AS-10, or sense ODN as control were introduced to the wells as described for the viability assay 12 hours before wounding the monolayer by scraping it with a sterile pipette tip. Medium was changed to RPMI 1640 supplemented with 5% FBS and fresh ODNs. The healing process was examined dynamically and recorded with a Nikon Coolpix 5000 digital camera with microscope adapter.

10) Boyden Chamber assay of migration

Cell migration assays were performed as previously described (Masood ANUP paper '99) except that 1 μ M AS-10 or AS-6 were added to the upper chamber. EGF (20 ng/ml) was used as chemoattractant in the lower chamber. Taxol at 10 ng/ml was used as a negative control.

11) In vivo studies

SCC15 (5×10^6 cells) were injected subcutaneously in the lower back of 5-week old male Balb/C Nu⁺/nu⁺ athymic mice. Treatment consisted of daily intraperitoneal injection of ODN (20 mg/kg in a total volume of 100 μ l) or diluent (PBS) begun the day following tumor cell implantation and continued for two weeks. Tumor growth in mice was measured as previously described (Masood CCR '01). Mice were sacrificed at the conclusion of the study. All mice were maintained in accord with the University of Southern California Animal Care and Use Committee guidelines governing the care of laboratory mice.

Example 6. Ephrin B2 Expression in Kaposi's Sarcoma Is Induced by Human Herpesvirus Type 8: Phenotype Switch from Venous to Arterial Endothelium

Kaposi's Sarcoma (KS) manifests as a multifocal angioproliferative disease, most commonly of the skin and mucus membranes, with subsequent spread to visceral organs (1) Hallmarks of the disease are angiogenesis, edema, infiltration of lymphomononuclear cells and growth of spindle-shaped tumor cells. Pathologically, established lesions exhibit an extensive vascular network of slit-like spaces. The KS vascular network is distinct from normal vessels in the lack of basement membranes and the abnormal spindle shaped

endothelial cell (tumor cell) lining these vessels. Defective vasculature results in an accumulation of the blood components including albumin, red and mononuclear cells in the lesions (1). The KS tumor is endothelial in origin; the tumor cells express many endothelial markers, including lectin binding sites for *Ulex europeaus* agglutinin-1 (UEA-1), CD34, EN-4, PAL-E (2) and the endothelial cell specific tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), VEGFR-3 (Flt-4), Tie-1 and Tie-2 (3, RM & PSG unpublished data). KS cells co-express lymphatic endothelial cell related proteins including LYVE and podoplanin (4).

The herpesvirus HHV-8 is considered the etiologic agent for the disease. In 1994 sequences of this new herpes virus were identified in KS tumor tissue (5), and subsequent molecular-epidemiology studies have shown that nearly all KS tumors contain viral genome. Sero-epidemiology studies show that HIV infected patients with KS have the highest prevalence of HHV-8 and secondly that those with HIV infection but no KS have increased risk of development of KS over the ensuing years if they are also seropositive for HHV-8 (6). Direct evidence for the role of HHV-8 in KS is the transformation of bone marrow endothelial cells after infection with HHV-8 (7). A number of HHV-8 encoded genes could contribute to cellular transformation (reviewed in 8). However, the most evidence has accumulated for the G-protein coupled receptor (vGPCR) in this role (9).

We investigated whether KS tumor cells are derived from arterial or venous endothelium. In addition, we investigated whether HHV-8 has an effect on expression of arterial or venous markers in a model of KS. KS tumor cells were found to express the ephrin B2 arterial marker. Further, ephrin B2 expression was induced by HHV-8 vGPCR in KS and endothelial cell lines. Ephrin B2 is a potential target for treatment of KS because inhibition of ephrin B2 expression or signaling was detrimental to KS cell viability and function.

A. KS tumors express Ephrin B2, but not EphB4

The highly vascular nature of KS lesions and the probable endothelial cell origin of the tumor cells prompted investigation of expression of EphB4 and ephrin B2 which are markers for venous and arterial endothelial cells, respectively. Ephrin B2, but not EphB4 transcripts were detected in tumor cells of KS biopsies by in situ hybridization (figure 45A). Comparison of the positive signal with ephrin B2 antisense probe and tumor cells as shown by H&E staining shows that ephrin B2 expression is limited to the areas of the biopsy that

contain tumor cells. The lack of signal in KS with EphB4 antisense probe is not due to a defect in the probe, as it detected transcripts in squamous cell carcinoma, which we have shown expresses this protein (18). Additional evidence for the expression of ephrin B2 in KS tumor tissue is afforded by the localization of EphB4/Fc signal to tumor cells, detected by FITC conjugated anti human Fc antibody. Because ephrin B2 is the only ligand for EphB4 this reagent is specific for the expression of ephrin B2 (figure 45B, left). An adjacent section treated only with the secondary reagent shows no specific signal. Two-color confocal microscopy demonstrated the presence of the HHV-8 latency protein, LANA1 in the ephrin B2 positive cells (Fig. 45C, left), indicating that it is the tumor cells, not tumor vessels, which are expressing this arterial marker. Staining of tumor biopsy with PECAM-1 antibody revealed the highly vascular nature of this tumor (Fig. 45C, right). A pilot study of the prevalence of this pattern of ephrin B2 and EphB4 expression on KS biopsies was conducted by RT-PCR analysis. All six samples were positive for ephrin B2, while only 2 were weakly positive for EphB4 (data not shown).

B. Infection of venous endothelial cells with HHV-8 causes a phenotype switch to arterial markers

We next asked whether HHV-8, the presumed etiologic agent for KS, could itself induce expression of ephrin B2 and repress EphB4 expression in endothelial cells. Co-culture of HUVEC and BC-1 lymphoma cells, which are productively infected with HHV-8, results in effective infection of the endothelial cells (16). The attached monolayers of endothelial cells remaining after extensive washing were examined for ephrin B2 and EphB4 by RT-PCR and immunofluorescence. HUVEC express EphB4 venous marker strongly at the RNA level, but not ephrin B2 (figure 46B). In contrast, HHV-8 infected cultures (HUVEC/BC-1 and HUVEC/BC-3) express ephrin B2, while EphB4 transcripts are almost absent.

Immunofluorescence analysis of cultures of HUVEC and HUVEC/HHV-8 for artery/vein markers and viral proteins was undertaken to determine whether changes in protein expression mirrored that seen in the RNA. In addition, cellular localization of the proteins could be determined. Consistent with the RT-PCR data HUVEC are ephrin B2 negative and EphB4 positive (Fig. 46A(a & m)). As expected they do not express any HHV-8 latency associated nuclear antigen (LANA1) (Fig. 46A(b, n)). Co-culture of BC-1 cells, which are productively infected with HHV-8, resulted in infection of HUVEC as shown by presence of viral proteins LANA1 and ORF59 (Fig. 46A(f, r)). HHV-8 infected HUVEC now

express ephrin B2 but not EphB4 (Fig. 46A(e, q, u), respectively). Expression of ephrin B2 and LANA1 co-cluster as shown by yellow signal in the merged image (Fig. 46A(h)). HHV-8 infected HUVEC positive for ephrin B2 and negative for Eph B4 also express the arterial marker CD148 (19) (Fig. 46A (j, v)). Expression of ephrin B2 and CD148 co-cluster as shown by yellow signal in the merged image (Fig. 46A(l)). Uninfected HUVEC expressing Eph B4 were negative for CD148 (not shown).

C. HHV-8 vGPCR induces ephrin B2 expression

To test whether individual viral proteins could induce the expression of ephrin B2 seen with the whole virus KS-SLK cells were stably transfected with HHV-8 LANA, or LANA Δ 440 or vGPCR. Western Blot of stable clones revealed a five-fold induction of ephrin B2 in KS-SLK transfected with vGPCR compared to SLK-LANA or SLK-LANA Δ 440 (Fig. 47A). SLK transfected with vector alone (pCEFL) was used as a control. SLK-vGPCR and SLK-pCEFL cells were also examined for ephrin B2 and Eph B4 expression by immunofluorescence in transiently transfected KS-SLK cells. Figure 47B shows higher expression of ephrin B2 in the SLK-vGPCR cells compared to SLK-pCEFL. No changes in Eph B4 were observed in SLK-vGPCR compared to SLK-pCEFL. This clearly demonstrates that SLK-vGPCR cells expressed high levels of ephrin B2 compared to SLK-pCEFL cells. This suggests that vGPCR of HHV-8 is directly involved in the induction of Ephrin B2 and the arterial phenotype switch in KS. Since we had shown that HHV-8 induced expression of ephrin B2 in HUVEC, we next asked if this could be mediated by a transcriptional effect. Ephrin B2 5'-flanking DNA-luciferase reporter plasmids were constructed as described in the Materials and Methods and transiently transfected into HUVECs. Ephrin B2 5'-flanking DNA sequences -2491/-11 have minimal activity in HUVEC cells (figure 47C). This is consistent with ephrin B2 being an arterial, not venous marker. However, we have noted that HUVEC in culture do express some ephrin B2 at the RNA level. Cotransfection of HHV-8 vGPCR induces ephrin B2 transcription approximately 10-fold compared to the control expression vector pCEFL. Roughly equal induction was seen with ephrin B2 sequences -2491/-11, -1242/-11, or -577/-11, which indicates that elements between -577 and -11 are sufficient to mediate the response to vGPCR, although maximal activity is seen with the -1242/-11 luciferase construct.

D. Expression of Ephrin B2 is regulated by VEGF and VEGF-C

We next asked whether known KS growth factors could be involved in the vGPCR-mediated induction of ephrin B2 expression. SLK-vGPCR cells were treated with neutralizing antibodies to oncostatin-M, IL-6, IL-8, VEGF or VEGF-C for 36 hr. Figure 48A shows that neutralization of VEGF completely blocked expression of ephrin B2 in SLK-vGPCR cells. A lesser, but significant decrease in ephrin B2 was seen neutralization of VEGF-C and IL-8. No appreciable effect was seen with neutralization of oncostatin-M or IL-6. To verify that VEGF and VEGF-C are integral to the induction of ephrin B2 expression we treated HUVEC with VEGF, VEGF-C or EGF. HUVECs were grown in EBM-2 media containing 5 % FBS with two different concentration of individual growth factor (10 ng, 100 ng/ml) for 48 h. Only VEGF-A or VEGF-C induced ephrin B2 expression in a dose dependent manner (Figure 48B). In contrast, EGF had no effect on expression of ephrin B2.

E. Ephrin B2 siRNA inhibits the expression of Ephrin B2 in KS

Three ephrin B2 siRNA were synthesized as described in the methods section. KS-SLK cells were transfected with siRNA and 48 h later ephrin B2 expression was determined by Western Blot. Ephrin B2 siRNAs 137 or 254 inhibited about 70% of ephrin B2 expression compared to control siRNA such as siRNA Eph B4 50 or siRNA GFP. Ephrin B2 63 siRNA was less effective than the above two siRNA Ephrin B2 (Figure 49A).

F. Ephrin B2 is necessary for full KS and EC viability, cord formation and in vivo angiogenesis activities

The most effective ephrin B2 siRNA (254) was then used to determine whether inhibiting expression of ephrin B2 has any effect on the growth of KS-SLK or HUVEC cells. The viability of KS-SLK cells was decreased by the same siRNAs that inhibited ephrin B2 protein levels (figure 49B). KS-SLK express high levels of ephrin B2 and this result shows maintenance of ephrin B2 expression is integral to cell viability in this setting. HUVECs do not express ephrin B2, except when stimulated by VEGF as shown in Fig. 48B. Ephrin B2 siRNA 264 dramatically reduced growth of HUVECs cultured with VEGF as the sole growth factor. In contrast, no significant effect was seen when HUVECs were cultured with IGF, EGF and bFGF. As a control, EphB4 siRNA 50 had no detrimental effect on HUVECs in either culture condition (figure 49C). In addition to inhibition of viability of KS and primary endothelial cells, EphB4-ECD inhibits cord formation in HUVEC and KS-SLK and in vivo angiogenesis in the MatrigelTM plug assay (Figure 50).

G. Methods and Materials

1) Cell lines and reagents

Human vascular endothelial cells (HUVEC) were from Clonetics (San Diego, CA) and were maintained in EGM-2 and EGM-2MV media respectively (Clonetics). T1 human fibroblast line was from Dr. Peter Jones, USC. BC-1 and BC-3 human pleural effusion lymphoma cell lines and monoclonal antibodies to LANA1 and ORF59 were the kind gift of Dr. Dharam Ablashi (Advanced Biotechnologies Inc., Columbia, MD). KS-SLK was isolated from a Classic Kaposi's sarcoma patient (15). Polyclonal antibodies to EphB4, ephrin B2, CD148, PECAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse EphB4/Fc' and monoclonal antibodies to human vascular endothelial growth factor (VEGF), VEGF-C, interleukin-(IL)6, IL-8 and oncostatin-M were purchased from R & D Systems (Minneapolis, MN). Expression vectors pKSvGPCR-CEFL and pCEFL were the kind gift of Dr. Enrique Mesri (Cornell University, New York, NY). Expression vectors for HHV-8 latency associated nuclear antigen (LANA) were kindly provided by Dr Matthew Rettig, Veteran's Administration Greater Los Angeles Healthcare System.

2) Collection and preparation of human tissue

Human cutaneous Kaposi's sarcoma biopsy material was obtained under local anesthesia with informed consent from patients at the LAC/USC Medical Center, using an IRB approved consent form. Biopsies were processed for either total RNA, paraffin blocks or frozen tissue blocks in OCT. Total RNA was extracted by homogenization in guanidine isothiocyanate, (RNAzol: Tel-Test, Inc., Friendswoods, TX). cDNAs were synthesized by reverse transcriptase using a random hexamer primer (Superscript II; Invitrogen, Carlsbad, CA).

3) Preparation of digoxigenin-labeled RNA probes

Ephrin B2 and EphB4 PCR products from the primers shown in Table 4 for in situ hybridization were cloned using the pGEM-T Easy system (Promega, Madison WI) according to the manufacturer's description using. The authenticity and insert orientation were confirmed by DNA sequencing. The pGEM-T Easy plasmids containing the PCR product of the human ephrin-B2 or EphB4 gene were linearized with *Spe* I or *Nco* I. Antisense or sense digoxigenin (DIG)-labeled RNA probes were transcribed from T7 or SP6

promoters by run-off transcription using a DIG RNA labeling kit (Roche, Indianapolis IN). RNA probes were quantitated by spot assay as described in the DIG RNA labeling kit instructions.

Table 4: Primers for Ephrin B2 and EphB4.

Gene	Primer sequence	Product Size (bp)
ISH Probe Primers		
ephrin B2	5' -TCC GTG TGG AGT ACT GCT G-3'	296
	5' -TCT GGT TTG GCA CAG TTG AG-3'	
EphB4	5' -CTT TGG AAG AGA CCC TGC TG-3'	297
	5' -AGA CGG TGA AGG TCT CCT TG-3'	
RT-PCR Primers		
ephrin B2	5' -AGA CAA GAG CCA TGA AGA TC-3'	200
	5' -GGA TCC CAC TTC GGA CCC GAG-3'	
EphB4	5' -TCA GGT CAC TGC ATT GAA CGG G-3'	400
	5' -AAC TCG CTC TCA TCC AGT T-3'	
β-actin	5' -GTG GGG CGC CCC AGG CAC CA-3'	546
	5' -CTC CTT AAT GTC ACG CAC GAT TTC-3'	

5

4) *In situ* hybridization

See above, e.g., Example 3.

5) Co-culture of HUVEC and BC-1

HUVEC cells were grown to 50-70% confluence in EGM-2 on gelatin-coated Labtech II 4-well chamber slides (Nalge Nunc International, Naperville, IL). Co-culture with BC-1 or BC-3 was essentially as described by Sakurada and coworkers (16). Briefly, BC-1 or BC-3 cells were pretreated with TPA (20 ng/ml) to induce virus for 48 hrs and then added to the HUVEC culture at a ratio of 10:1 for cocultivation for two days. The HUVECs were washed extensively with PBS to remove the attached BC-1 or BC-3 cells.

6) Preparation of cDNA and RT-PCR

The TITANIUM™ One-Step RT-PCR kit (Clontech, Palo Alto, CA) was used for RT-PCR from 1×10^5 cells. Primer pairs for amplification of EphB4, ephrin B2 and β -actin are shown in Table 4. Each PCR cycle consisted of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The samples were amplified for 30 cycles. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

7) Cell viability assay

KS-SLK cells were seeded at a density of 1×10^4 per well in 48-well plates on day 0 in appropriate growth media containing 2% fetal calf serum (FCS). On the following day, the media was changed and cells were treated with 0, 10 or 100 nM siRNA. On day 3, viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (17).

8) Immunofluorescence studies

Cells cultured on Labtech II 4-well chamber slides or frozen sections of KS biopsy material were fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline pH 7.4 (PBS) for 30 min. The slides were rinsed twice in PBS and preincubated with blocking buffer (0.2% Triton-X100, 1% BSA in PBS) for 20 min, followed by incubation with antibodies to EphB4, ephrin B2, CD148, LANA1 or ORF59 (1:100 dilution in PBS) in blocking buffer at 4 °C for 16 hr. After washing three times, the slides were incubated with the appropriate fluorescein or rhodamine-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), washed extensively with PBS and mounted with Vectasheild antifade mounting solution (Vector Laboratories, Burlingame, CA). Images were obtained using a Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments Inc., Sterling Heights, MI) digital imaging system.

Immunofluorescence detection of EphrinB2 with EPHB4-Fc was done as follows. Frozen sections fixed in 4% paraformaldehyde and blocked with 20% FBS were incubated with 5 μ g/ml EphB4/Fc (R&D Systems) for 1 h at RT. Sections were then incubated with 10 μ g/ml rabbit anti-human IgG-FITC in PBS (Jackson ImmunoResearch Laboratories West

Grove, PA) at RT for 1 hour. Nuclei were counterstained with DAPI and sections mounted as above. Human Fc (Jackson ImmunoResearch) was used as the negative control.

9) Western Blot

Crude cell lysates were prepared, quantitated, fractionated and transferred to
5 membranes as described previously (17). Membranes were blocked with 5% non-fat milk prior to incubation with antibody to ephrin B2 (1:5000 dilution) at 4 °C, for 16 h. Secondary antibody (1:100,000 dilution) conjugated with horseradish peroxidase was applied for 1 h at 25 °C. The membranes were developed using the SuperSignal West Femto Maximum
10 sensitivity chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Membranes were stripped using Restore™ Western Blot Stripping Buffer (Pierce) and reprobed with EphB4 or β -actin.

10) Cord formation assay

Matrigel™ Basement Membrane Matrix (BD Biosciences Discovery Labware,
Bedford, MA) was mixed with growth medium (3:1) on ice and 0.5 ml liquid placed in 24-
15 well plates. Incubation of plates at 37 °C for 15 min caused Matrigel™ polymerization. HUVEC or KS-SLK in exponential phase growth were treated with 2 or 8 μ g/ml EphB4-ECD or PBS as control for 16 h prior to trypsinizing and plating on the Matrigel™. Culture on
Matrigel™ was continued in the presence of recombinant fusion proteins for 6 h. Cultures
were fixed in 4% paraformaldehyde for 30 min and evaluated by inverted phase-contrast
20 photomicroscopy.

11) Synthesis of Ephrin B2 and EphB4 siRNA by in vitro transcription

The Silencer™ siRNA construction kit (Ambion, Austin TX) was used to synthesize
siRNA to ephrin B2 and EphB4. Briefly, three 21 bp target sequences comprising 19 bp
downstream of a 5'-AA dinucleotide were identified in the ephrin B2 cDNA (Accession
25 number NM_004093) that showed no significant homology to other sequences in the GenBank database. Sense and antisense siRNA 29-mer DNA oligonucleotide templates were synthesized at the USC Norris Microchemical Core Facility. Antisense template
corresponded to the target sequence followed by 8 bp addition (5'-CCTGTCTC-3') at the 3'
end complementary to the T7 promoter primer provided with the Silencer SiRNA
30 Construction Kit. Sense template comprised 5'-AA followed by the complement of the target

19 bp, then the T7 8 bp sequence as above. In separate reactions, the two siRNA oligonucleotide templates were hybridized to a T7 promoter primer. The 3' ends of the hybridized oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The dsRNA consisted of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs. The leader sequences were removed by digesting the dsRNA with a single-stranded specific ribonuclease. The DNA template was removed at the same time by treatment with RNase free deoxyribonuclease.

The resulting siRNAs were purified by glass fiber filter binding to remove excess nucleotides, short oligomers, proteins, and salts in the reaction. End product double-stranded 21mer siRNAs are shown in Table 5. Similarly, an EphB4 and green fluorescence protein (GFP) siRNAs were synthesized.

Table 5: siRNAs of ephrin B2 and EphB4.

ephrin B2 264	5' -GCAGACAGAU GCACUAUUAUU-3' 3' -UUCGUCUGUCUACGUGAUAAU-5'
ephrin B2 63:	5' -CUGCGAUUUC CAAAU CGAUUU-3' 3' -UUGACGCUAAAGGUUUAGCUA-5'
ephrin B2 137:	5' -GGACUGGUACUAUACCCACUU-3' 3' -UUCCUGACCAUGAU AUGGGUG-5'
Eph B4 50:	5' -GAGACCCUGCUGAACACAAUU-3' 3' -UUCUCUGGGACGACUUGUGUU-5'
GFP	5' -CGCUGACCCUGAAGUUCAUUU-3' 3' -UUGCGACUGGGACUUC AAGUA-5'

12) Transfection of Ephrin B2 or EphB4 siRNA

HUVEC were seeded on eight-well chamber slides coated with fibronectin and grown overnight in EGM-2 (Cambrex, Walkersville, MD). 16 h later media was replaced either with EBM-2 supplemented with 5% fetal calf serum (FCS) and EGM-2 BulletKit supplements bFGF, hEGF and R³-IGF-I at the concentrations provided by the manufacturer, or EBM-2 supplemented with 5% FCS and 10 ng/ml rhVEGF (R&D Systems). After 2 h incubation at 37 °C, the cells were transfected using Lipofectamine 2000 (1 µg/ml; Invitrogen) and 10 nM

specific siRNAs in Opti-MEM-1 serum-free medium (Invitrogen). Following transfection for 2 hr in Opti-MEM-1, media supplemented as above was replaced in the appropriate wells. After 48 hrs, the cells were stained with crystal violet and immediately photographed at 10X magnification.

5 13) Construction of ephrin B2 reporter plasmids

Human ephrin B2 5'-flanking DNA from -2491 to -11 with respect to the translation start site was amplified from BACPAC clone RP11-297I6 (BacPac Resources, Children's Hospital, Oakland, CA) using the Advantage GC Genomic PCR kit (Clontech Palo Alto, CA) to overcome the large tracts of CG-rich sequence in the target area. Primers were designed to
10 contain *Mlu*I sites for cloning. Amplified product was digested with *Mlu*I, gel purified and ligated into the *Mlu*I site in the multiple cloning site of pGL3Basic (Promega, Madison, WI). Orientation of the resulting clones was confirmed by restriction digest analysis. The correct clone was designated pEFNB2_{-2491/-11}luc. Digestion of this clone with either *Kpn*I or *Sac*I followed by recircularization yielded pEFNB2_{-1242/-11}luc and pEFNB2_{-577/-11}luc, respectively.
15 Plasmid DNAs used for transient transfections were purified using a Mega Prep kit (QIAGEN, Valencia, CA).

14) Transient transfection

HUVEC cells (0.8×10^4 cells/well in 24 well plates) maintained in EGM-2 media were transiently co-transfected with 0.5 µg/well ephrin B2 promoter-luciferase constructs
20 together with 50 ng/well either pCEFL or pKSvGPCR-CEFL, using Superfect reagent (QIAGEN) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection and lysed with Luciferase cell lysis buffer (Promega). Luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase was normalized to protein, because pCEFL-vGPCR induced the
25 expression of β-galactosidase from pCMV-Sport-βgal (Invitrogen).

15) Construction and purification of EphB4 extra cellular domain (ECD) protein

See above, e.g., Example 1.

Example 7. Expression of EphB4 in Bladder cancer: a candidate target for therapy

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

5 Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

10 Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μ M).

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

15 Example 8. Inhibition of EphB4 Gene Expression by EphB4 antisense probes and RNAi probes

Cell lines expressing EphB4 were treated with the synthetic phosphorothioate modified oligonucleotides and harvested after 24 hr. Cell lysates were prepared and probed by western blot analysis for relative amounts of EphB4 compared to untreated control cells.

20 Studies on inhibition of cell proliferation were done in HNSCC cell lines characterized to express EphB4. Loss of cell viability was shown upon knock-down of EphB4 expression. Cells were treated in vitro and cultured in 48-well plates, seeded with 10 thousand cells per well. Test compounds were added and the cell viability was tested on day 3. The results on EphB4 antisense probes were summarized below in Table 6. The results on EphB4 RNAi probes were summarized below in Table 7.

25

Table 6. Inhibition of EphB4 Gene Expression by EphB4 antisense probes

Name	Sequence 5' \rightarrow 3'	position	Inhibition of EphB4 Expression	Percent reduction in viability
Eph B4 169	TCA GTA CTG CGG GGC CGG TCC	(2944-2963)	++	36
Eph B4 168	TCC TGT CCC ACC CGG GGT TC	(2924-2943)	++	51
Eph B4 167	CCG GCT TGG CCT GGG ACT TC	(2904-2923)	+++	66
Eph B4 166	ATG TGC TGG ACA CTG GCC AA	(2884-2903)	++++	70
Eph B4 165	GAT TTT CTT CTG GTG TCC CG	(2864-2883)	++++	75
Eph B4 164	CCA GAG TGA CTC CGA TTC GG	(2844-2863)	++	40
Eph B4 163	AGC AGG TCC TCA GCA GAG AT	(2824-2843)	++++	66
Eph B4 162	CTG GCT GAC CAG CTC GAA GG	(2804-2823)		25

Eph B4 161	AGC CAA AGC CAG CGG CTG CG	(2784-2803)	+	33
Eph B4 160	AAA CTT TCT TCG TAT CTT CC	(2763-2783)	+	25
Eph B4 159	CAT TTT GAT GGC CCG AAG CC	(2743-2762)	++	40
Eph B4 158	ACT CGC CCA CAG AGC CAA AA	(2723-2742)		30
Eph B4 157	GCT GAG TAG TGA GGC TGC CG	(2703-2722)	+	25
Eph B4 156	CTG GTC CAG GAG AGG GTG TG	(2683-2702)	++	30
Eph B4 155	AGG CCC CGC CAT TCT CCC GG	(2663-2682)		25
Eph B4 154	GCC ACG ATT TTG AGG CTG GC	(2643-2662)	++	40
Eph B4 153	GGG GTT CCG GAT CAT CTT GT	(2623-2642)	++	35
Eph B4 152	CCA GGG CGC TGA CCA CCT GG	(2603-2622)	+	30
Eph B4 151	GGG AAG CGG GGC CGG GCA TT	(2583-2602)	+	25
Eph B4 150	CCG GTC TTT CTG CCA ACA GT	(2563-2582)	++	25
Eph B4 149	CCA GCA TGA GCT GGT GGA GG	(2543-2562)	++	20
Eph B4 148	GAG GTG GGA CAG TCT GGG GG	(2523-2542)	+	30
Eph B4 147	CGG GGG CAG CCG GTA GTC CT	(2503-2522)	++	40
Eph B4 146	GTT CAA TGG CAT TGA TCA CG	(2483-2502)	++++	70
Eph B4 145	TCC TGA TTG CTC ATG TCC CA	(2463-2482)	++++	80
Eph B4 144	GTA CGG CCT CTC CCC AAA TG	(2443-2462)	+++	60
Eph B4 143	ACA TCA CCT CCC ACA TCA CA	(2423-2442)	++++	80
Eph B4 142	ATC CCG TAA CTC CAG GCA TC	(2403-2422)	++	40
Eph B4 141	ACT GGC GGA AGT GAA CTT CC	(2383-2402)	+++	50
Eph B4 140	GGA AGG CAA TGG CCT CCG GG	(2363-2382)	++	45
Eph B4 139	GCA GTC CAT CGG ATG GGA AT	(2343-2362)	++++	70
Eph B4 138	CTT TCC TCC CAG GGA GCT CG	(2323-2342)	++++	70
Eph B4 137	TGT AGG TGG GAT CGG AAG AG	(2303-2322)	++	40
Eph B4 136	TTC TCC TCC AGG AAT CGG GA	(2283-2302)	++	35
Eph B4 135	AAG GCC AAA GTC AGA CAC TT	(2263-2282)	++++	60
Eph B4 134	GCA GAC GAG GTT GCT GTT GA	(2243-2262)	++	50
Eph B4 133	CTA GGA TGT TGC GAG CAG CC	(2223-2242)	++	40
Eph B4 132	AGG TCT CGG TGG ACG TAG CT	(2203-2222)	++	40
Eph B4 131	CAT CTC GGC AAG GTA CCG CA	(2183-2202)	+++	50
Eph B4 130	TGC CCG AGG CGA TGC CCC GC	(2163-2182)	++	50
Eph B4 129	AGC ATG CCC ACG AGC TGG AT	(2143-2162)	++	50
Eph B4 128	GAC TGT GAA CTG TCC GTC GT	(2123-2142)	++	50
Eph B4 127	TTA GCC GCA GGA AGG AGT CC	(2103-2122)	+++	60
Eph B4 126	AGG GCG CCG TTC TCC ATG AA	(2083-2102)	++	50
Eph B4 125	CTC TGT GAG AAT CAT GAC GG	(2063-2082)	++++	80
Eph B4 124	GCA TGC TGT TGG TGA CCA CG	(2043-2062)	++++	70
Eph B4 123	CCC TCC AGG CGG ATG ATA TT	(2023-2042)	++	50
Eph B4 122	GGG GTG CTC GAA CTG GCC CA	(2003-2022)	++++	80
Eph B4 121	TGA TGG AGG CCT CGC TCA GA	(1983-2002)	++	50
Eph B4 120	AAC TCA CGC CGC TGC CGC TC	(1963-1982)	++	40
Eph B4 119	CGT GTA GCC ACC CTT CAG GG	(1943-1962)	++++	75
Eph B4 118	TCT TGA TTG CCA CAC AGC TC	(1923-1942)	++++	80
Eph B4 117	TCC TTC TTC CCT GGG GCC TT	(1903-1922)	++++	70
Eph B4 116	GAG CCG CCC CCG GCA CAC CT	(1883-1902)	++	50
Eph B4 115	CGC CAA ACT CAC CTG CAC CA	(1863-1882)	++++	60
Eph B4 114	ATC ACC TCT TCA ATC TTG AC	(1843-1862)	++++	65
Eph B4 113	GTA GGA GAC ATC GAT CTC TT	(1823-1842)	++++	90
Eph B4 112	TTG CAA ATT CCC TCA CAG CC	(1803-1822)	++++	70
Eph B4 111	TCA TTA GGG TCT TCA TAA GT	(1783-1802)	++++	70
Eph B4 110	GAA GGG GTC GAT GTA GAC CT	(1763-1782)	++++	80
Eph B4 109	TAG TAC CAT GTC CGA TGA GA	(1743-1762)	++	50
Eph B4 108	TAC TGT CCG TGT TTG TCC GA	(1723-1742)	++	45
Eph B4 107	ATA TTC TGC TTC TCT CCC AT	(1703-1722)	++++	70
Eph B4 106	TGC TCT GCT TCC TGA GGC AG	(1683-1702)	++++	70
Eph B4 105	AGA ACT GCG ACC ACA ATG AC	(1663-1682)	++	40
Eph B4 104	CAC CAG GAC CAG GAC CAC AC	(1643-1662)	++++	70

Eph B4 103	CCA CGA CTG CCG TGC CCG CA	(1623-1642)	++	40
Eph B4 102	ATC AGG GCC AGC TGC TCC CG	(1603-1622)	+++	50
Eph B4 101	CCA GCC CTC GCT CTC ATC CA	(1583-1602)	++++	80
Eph B4 100	GTT GGG TCT GGC TGT GAT GT	(1563-1582)	++++	80
Eph B4 99	TCC TGG CCG AAG GGC CCG TA	(1543-1562)	++	35
Eph B4 98	GCC GGC CTC AGA GCG CGC CC	(1523-1542)	++	50
Eph B4 97	GTA CCT GCA CCA GGT AGC TG	(1503-1522)	++++	80
Eph B4 96	GCT CCC CGC TTC AGC CCC CG	(1483-1502)	++	50
Eph B4 95	CAG CTC TGC CCG GTT TTC TG	(1463-1482)	++	50
Eph B4 94	ACG TCT TCA GGA ACC GCA CG	(1443-1462)	++++	80
Eph B4 93	CTG CTG GGA CCC TCG GCG CC	(1423-1442)	++	40
Eph B4 92	CTT CTC ATG GTA TTT GAC CT	(1403-1422)	++++	80
Eph B4 91	CGT AGT CCA GCA CAG CCC CA	(1383-1402)	++++	85
Eph B4 90	CTG GGT GCC CGG GGA ACA GC	(1363-1382)	+++	50
Eph B4 89	CCA GGC CAG GCT CAA GCT GC	(1343-1462)	++++	70
Eph B4 88	TGG GTG AGG ACC GCG TCA CC	(1323-1342)	++	40
Eph B4 87	CGG ATG TCA GAC ACT GCA GG	(1303-1322)	++++	60
Eph B4 86	AGG TAC CTC TCG GTC AGT GG	(1283-1302)	++	50
Eph B4 85	TGA CAT TGA CAG GCT CAA AT	(1263-1282)	++++	80
Eph B4 84	GGG ACG GGC CCC GTG GCT AA	(1243-1262)	++	50
Eph B4 83	GGA GGA TAC CCC GTT CAA TG	(1223-1242)	+++	60
Eph B4 82	CAG TGA CCT CAA AGG TAT AG	(1203-1222)	++++	70
Eph B4 81	GTG AAG TCA GGA CGT AGC CC	(1183-1202)	+++	60
Eph B4 80	TCG AAC CAC CAC CCA GGG CT	(1163-1182)	+++	50
Eph B4 79	CCA CCA GGT CCC GGG GGC CG	(1143-1162)	++	40
Eph B4 78	GGG TCA AAA GTC AGG TCT CC	(1123-1142)	++++	70
Eph B4 77	CCC GCA GGG CGC ACA GGA GC	(1103-1122)	+++	60
Eph B4 76	CTC CGG GTC GGC ACT CCC GG	(1083-1102)	+++	60
Eph B4 75	CAG CGG AGG GCG TAG GTG AG	(1063-1082)	++	40
Eph B4 74	GTC CTC TCG GCC ACC AGA CT	(1043-1062)	++	50
Eph B4 73	CCA GGG GGG CAC TCC ATT CC	(1023-1042)	++	50
Eph B4 72	AGG TGC AGG GAG GAG CCG TT	(1003-1022)	++++	70
Eph B4 71	CAG GCG GGA AAC CAC GCT CC	(983-1002)	++	40
Eph B4 70	GCG GAG CCG AAG GAG GGG TG	(963-982)	+++	50
Eph B4 69	GTG CAG GGT GCA CCC CGG GG	(943-962)	+++	50
Eph B4 68	GTC TGT GCG TGC CCG GAA GT	(923-942)	++	40
Eph B4 67	ACC CGA CGC GGC ACT GGC AG	(903-922)	++	40
Eph B4 66	ACG GCT GAT CCA ATG GTG TT	(883-902)	++	50
Eph B4 65	AGA GTG GCT ATT GGC TGG GC	(863-882)	++++	60
Eph B4 64	ATG GCT GGC AGG ACC CTT CT	(843-862)	++++	80
Eph B4 63	CCT GAC AGG GGC TTG AAG GT	(823-842)	++++	80
Eph B4 62	GCC CTG GGC ACA GGC TCG GC	(803-822)	+++	70
Eph B4 61	ACT TGG TGT TCC CCT CAG CT	(783-802)	++++	80
Eph B4 60	GCC TCG AAC CCC GGA GCA CA	(763-782)	+++	50
Eph B4 59	GCT GCA GCC CGT GAC CGG CT	(743-762)	+++	50
Eph B4 58	GTT CGG CCC ACT GGC CAT CC	(723-742)	++	45
Eph B4 57	TCA CGG CAG TAG AGG CTG GG	(703-722)	+++	70
Eph B4 56	GCT GGG GCC AGG GGC GGG GA	(683-702)	++	50
Eph B4 55	CGG CAT CCA CCA CGC AGC TA	(663-682)	++	50
Eph B4 54	CCG GCC ACG GGC ACA ACC AG	(643-662)	++	50
Eph B4 53	CTC CCG AGG CAC AGT CTC CG	(623-642)	+++	50
Eph B4 52	GGA ATC GAG TCA GGT TCA CA	(603-622)	++++	90
Eph B4 51	GTC AGC TGG GCG CAC TTT TT	(583-602)	+++	70
Eph B4 50	GTA GAA GAG GTG CAG GGA TA	(563-582)	++++	80
Eph B4 49	GCA GGG CCA TGC AGG CAC CC	(543-562)	++++	80
Eph B4 48	TGG TCC TGG AAG GCC AGG TA	(523-542)	++++	90
Eph B4 47	GAA GCC AGC CTT GCT GAG CG	(503-522)	++++	80
Eph B4 46	GTC CCA GAC GCA GCG TCT TG	(483-502)	++	40

Eph B4 45	ACA TTC ACC TTC CCG GTG GC	(463-482)	+++	50
Eph B4 44	CTC GGC CCC AGG GCG CTT CC	(443-462)	++	50
Eph B4 43	GGG TGA GAT GCT CCG CGG CC	(423-442)	+++	60
Eph B4 42	ACC GTG TCC ACC TTG ATG TA	(403-422)	++++	80
Eph B4 41	GGG GTT CTC CAT CCA GGC TG	(383-402)	++++	80
Eph B4 40	GCG TGA GGG CCG TGG CCG TG	(363-382)	++	50
Eph B4 39	TCC GCA TCG CTC TCA TAG TA	(343-362)	+++	60
Eph B4 38	GAA GAC GGT GAA GGT CTC CT	(323-342)	++++	80
Eph B4 37	TGC AGG AGC GCC CAG CCC GA	(303-322)	+++	50
Eph B4 36	GGC AGG GAC AGG CAC TCG AG	(283-302)	+++	45
Eph B4 35	CAT GGT GAA GCG CAG CGT GG	(263-282)	++	50
Eph B4 34	CGT ACA CGT GGA CGG CGC CC	(243-262)	++	40
Eph B4 33	CGC CGT GGG ACC CAA CCT GT	(223-242)	+++	60
Eph B4 32	GCG AAG CCA GTG GGC CTG GC	(203-222)	++++	70
Eph B4 31	CCG GGG CAC GCT GCA CGT CA	(183-202)	+++	60
Eph B4 30	CAC ACT TCG TAG GTG CGC AC	(163-182)	+++	70
Eph B4 29	GCT GTG CTG TTC CTC ATC CA	(143-162)	++++	80
Eph B4 28	GGC CGC TCA GTT CCT CCC AC	(123-142)	++	40
Eph B4 27	TGC CCG TCC ACC TGA GGG AA	(103-122)	++	50
Eph B4 26	TGT CAC CCA CTT CAG ATC AG	(83-102)	++++	70
Eph B4 25	CAG TTT CCA ATT TTG TGT TC	(63-82)	++++	70
Eph B4 24	AGC AGG GTC TCT TCC AAA GC	(43-62)	++++	80
Eph B4 23	TGC GGC CAA CGA AGC CCA GC	(23-42)	++	50
Eph B4 22	AGA GCA GCA CCC GGA GCT CC	(3-22)	+++	50
Eph B4 21	AGC AGC ACC CGG AGC TCC AT	(1-20)	+++	50
Additional antisense probes described in the specification				
EphB4 AS-1	GTG CAG GGA TAG CAG GGC CAT	(552-572)		
EphB4 AS-2	AAG GAG GGG TGG TGC ACG GTG	(952-972)		
EphB4 AS-3	TTC CAG GTG CAG GGA GGA GCC	(1007-1027)		
EphB4 AS-4	GTG GTG ACA TTG ACA GGC TCA	(1263-1285)		
EphB4 AS-5	TCT GGC TGT GAT GTT CCT GGC	(1555-1575)		
EphB4 AS-6	GCC GCT CAG TTC CTC CCA	(123-140)		
EphB4 AS-7	TGA AGG TCT CCT TGC AGG	(316-333)		
EphB4 AS-8	CGC GGC CAC CGT GTC CAC CTT	(408-428)		
EphB4 AS-9	CTT CAG GGT CTT GAT TGC CAC	(1929-1949)		
EphB4 AS-10	ATG GAG GCC TCG CTC AGA AA	(1980-1999)		
EphB4 AS-11	CAT GCC CAC GAG CTG GAT GAC	(2138-2158)		

Table 7. Inhibition of EphB4 Gene Expression by EphB4 RNAi probes

RNAi	EphB4 RNAi sequence		Inhibition of EphB4 Expression	Percent reduction in viability
1	446	aaattggaaactgctgatctg 466		
2	447	aattggaaactgctgatctga 467	+++	70
3	453	aaactgctgatctgaagtggg 473	++++	70
4	454	aactgctgatctgaagtgggt 474	+++	80
5	854	aatgtcaagacgctgctgctg 874	+++	65
6	467	aagtgggtgacattccctcag 487	+	35
7	848	aagggtgaatgtcaagacgctg 868	++	50

8	698	aaggagaccttcaccgtcttc	718	+++	75
9	959	aaaaagtgcgcccagctgact	979	+	40
10	1247	aatagccactctaaccatt	1267	++	50
11	1259	aacaccattggatcagccgtc	1279	++	50
12	1652	aatgtcaccactgaccgagag	1672	+	35
13	1784	aaataccatgagaagggcgcc	1804	+++	65
14	1832	aagacgtcagaaaaccgggca	1852	+	30
15	1938	aacatcacagccagacccaac	19	++	50
16	2069	aagcagagcaatgggagagaa	2089	++++	75
17	2078	aatgggagagaagcagaatat	2098	+++	65
18	2088	aagcagaatattcggacaaac	2108	+++	70
19	2094	aatattcggacaaacacggac	2114	++	40
20	2105	aaacacggacagtatctcatc	2125	++	50
21	2106	aacacggacagtatctcatcg	2126	+	35
22	2197	aaaagagatcgatgtctccta	2217	+++	65
23	2174	aatgaggctgtgaggggaattt	2194	++	50
24	2166	aagaccctaagagggtgtga	2186	++	50
25	2198	aaagagatcgatgtctcctac	2218	+++	55
26	2199	aagagatcgatgtctcctacg	2219	+++	70
27	2229	aagaggtgattggtgcagggtg	2249	+	33
28	2222	aagattgaagaggtgattggt	2242	+	30
29	2429	aacagcatgcccgtcatgatt	2449	++	40
30	2291	aagaaggagagctgtgtggca	2311	+++	50
31	2294	aaggagagctgtgtggcaatc	2314	+++	60
32	2311	aatcaagaccctgaaggggtg	2331	+++	70
33	2497	aaacgacggacagttcacagt	2517	+	35
34	2498	aacgacggacagttcacagtc	2518	+	40
35	2609	aacatcctagtcaacagcaac	2629	++	50
36	2621	aacagcaacctcgtctgcaa	2641	+	35
37	2678	aactcttccgatccacactac	2698	++	50
38	2640	aagtgtctgactttggccttt	2660	+++	70
39	2627	aacctcgtctgcaaagtgtct	2647	++	50
40	2639	aaagtgtctgactttggcctt	2659	+	25
41	2852	aatcaggacgtgatcaatgcc	2872	+++	75
42	2716	aaagattcccatccgatggac	2736	++	50
43	2717	aagattcccatccgatggact	2737	++	60
44	2762	aagttcacttccgccagtgat	2782	+++	70
45	3142	aagatacgaagaaagtttcgc	3162	++	50
46	3136	aatgggaagatacgaagaaag	3156	+++	66
47	2867	aatgccattgaacaggactac	2887		
48	3029	aaaatcgtggcccgggagaat	3049	+	33
49	3254	aaaatcttggccagtgtccag	3274	++	50

50	3255	aaatcttggccagtgtccagc	3275	+++	75
51	3150	aagaaagtttgcgagccgctg	3170	+++	80
52	3251	aagaaaatcttggccagtgtc	3271	++	50
53	3256	aatcttggccagtgtccagca	3276	++	50
Additional RNAi probes described in the specification					
Eph B4 50		gagaccugcugaacacaaau			
Eph B4 472		ggugaaugucaagacgcuguu			
Eph B4 1562		caucacagccagacccaacuu			
siRNA 2303		cucuuccgaucccaccuacuu			
Eph B4 2302		cucuuccgaucccaccuacuu			

Example 9. Inhibition of Ephrin B2 Gene Expression by Ephrin B2 antisense probes and RNAi probes

KS SLK, a cell line expressing endogenous high level of ephrin B2. Cell viability was tested using fixed dose of each oligonucleotide (50M). Gene expression downregulation was done using cell line 293 engineered to stably express full-length ephrin B2. KS SLK expressing EphrinB2 were also used to test the viability in response to RNAi probes tested at the fixed dose of 50 nM. Protein expression levels were measured using 293 cells stably expressing full-length EphrinB2, in cell lysates after 24 hr treatment with fixed 50 nM of RNAi probes.

The results on Ephrin B2 antisense probes were summarized below in Table 8. The results on Ephrin B2 RNAi probes were summarized below in Table 9.

Table 8. Ephrin B2 antisense ODNs.

	sequence	Coding region	Percent reduction in viability	Inhibition of Ephrin B2 Expression
Ephrin AS-51	TCA GAC CTT GTA GTA AAT GT	(983-1002)	35	++
Ephrin AS-50	TCG CCG GGC TCT GCG GGG GC	(963-982)	50	+++
Ephrin AS-49	ATC TCC TGG ACG ATG TAC AC	(943-962)	45	++
Ephrin AS-48	CGG GTG CCC GTA GTC CCC GC	(923-942)	35	++
Ephrin AS-47	TGA CCT TCT CGT AGT GAG GG	(903-922)	40	+++
Ephrin AS-46	CAG AAG ACG CTG TCC GCA GT	(883-902)	40	++
Ephrin AS-45	CCT TAG CGG GAT GAT AAT GT	(863-882)	35	++
Ephrin AS-44	CAC TGG GCT CTG AGC CGT TG	(843-862)	60	+++
Ephrin AS-43	TTG TTG CCG CTG CGC TTG GG	(823-842)	40	++
Ephrin AS-	TGT GGC CAG TGT GCT GAG CG	(803-822)	40	++

42				
Ephrin AS-41	ACA GCG TGG TCG TGT GCT GC	(783-802)	70	+++
Ephrin AS-40	GGC GAG TGC TTC CTG TGT CT	(763-782)	80	++++
Ephrin AS-39	CCT CCG GTA CTT CAG CAA GA	(743-762)	50	+++
Ephrin AS-38	GGA CCA CCA GCG TGA TGA TG	(723-742)	60	+++
Ephrin AS-37	ATG ACG ATG AAG ATG ATG CA	(703-722)	70	+++
Ephrin AS-36	TCC TGA AGC AAT CCC TGC AA	(683-702)	60	+++
Ephrin AS-35	ATA AGG CCA CTT CGG AAC CG	(663-682)	45	++
Ephrin AS-34	AGG ATG TTG TTC CCC GAA TG	(643-662)	50	+++
Ephrin AS-33	TCC GGC GCT GTT GCC GTC TG	(623-642)	75	+++
Ephrin AS-32	TGC TAG AAC CTG GAT TTG GT	(603-622)	60	+++
Ephrin AS-31	TTT ACA AAG GGA CTT GTT GT	(583-602)	66	+++
Ephrin AS-30	CGA ACT TCT TCC ATT TGT AC	(563-582)	50	++
Ephrin AS-29	CAG CTT CTA GTT CTG GAC GT	(543-562)	50	+++
Ephrin AS-28	CTT GTT GGA TCT TTA TTC CT	(523-542)	70	+++
Ephrin AS-27	GGT TGA TCC AGC AGA ACT TG	(503-522)	65	+++
Ephrin AS-26	CAT CTT GTC CAA CTT TCA TG	(483-502)	75	+++
Ephrin AS-25	AGG ATC TTC ATG GCT CTT GT	(463-482)	60	+++
Ephrin AS-24	CTG GCA CAC CCC TCC CTC CT	(443-462)	45	++
Ephrin AS-23	GGT TAT CCA GGC CCT CCA AA	(423-442)	50	+++
Ephrin AS-22	GAC CCA TTT GAT GTA GAT AT	(403-422)	50	+++
Ephrin AS-21	AAT GTA ATA ATC TTT GTT CT	(383-402)	60	+++
Ephrin AS-20	TCT GAA ATT CTA GAC CCC AG	(363-382)	60	+++
Ephrin AS-19	AGG TTA GGG CTG AAT TCT TG	(343-362)	75	+++
Ephrin AS-18	AAA CTT GAT GGT GAA TTT GA	(323-342)	60	+++
Ephrin AS-17	TAT CTT GGT CTG GTT TGG CA	(303-322)	50	++
Ephrin AS-16	CAG TTG AGG AGA GGG GTA TT	(283-302)	40	++
Ephrin AS-15	TTC CTT CTT AAT AGT GCA TC	(263-282)	66	+++
Ephrin AS-14	TGT CTG CTT GGT CTT TAT CA	(243-262)	70	++++
Ephrin AS-13	ACC ATA TAA ACT TTA TAA TA	(223-242)	50	+++
Ephrin AS-12	TTC ATA CTG GCC AAC AGT TT	(203-222)	50	+++
Ephrin AS-11	TAG AGT CCA CTT TGG GGC AA	(183-202)	70	++++
Ephrin AS-10	ATA ATA TCC AAT TTG TCT CC	(163-182)	70	++++
Ephrin AS-	TAT CTG TGG GTA TAG TAC CA	(143-162)	80	++++

9				
Ephrin AS-8	GTC CTT GTC CAG GTA GAA AT	(123-142)	60	+++
Ephrin AS-7	TTG GAG TTC GAG GAA TTC CA	(103-122)	80	++++
Ephrin AS-6	ATA GAT AGG CTC TAA AAC TA	(83-102)	70	+++
Ephrin AS-5	TCG ATT TGG AAA TCG CAG TT	(63-82)	50	+++
Ephrin AS-4	CTG CAT AAA ACC ATC AAA AC	(43-62)	80	++++
Ephrin AS-3	ACC CCA GCA GTA CTT CCA CA	(23-42)	85	++++
Ephrin AS-2	CGG AGT CCC TTC TCA CAG CC	(3-22)	70	+++
Ephrin AS-1	GAG TCC CTT CTC ACA GCC AT	(1-20)	80	++++

Table 9. Ephrin B2 RNAi probes.

RNAi Sequence and homology with other human genes.			Percent reduction in viability	Inhibition of Ephrin B2 Expression	RNAi no.
89	aactgcgatttccaaatcgat	109	80	++++	1
141	aactccaaatttctacctgga	161	70	++++	2
148	aatttctacctggacaaggac	168	75	+++	3
147	aaatttctacctggacaagga	167	60	+++	4
163	aaggactggtactataccac	183	40	++	5
217	aagtggactctaaaactgttg	237	80	++++	6
229	aaactgttggccagtatgaat	249	50	+++	7
228	aaaactgttggccagtatgaa	248	80	++++	8
274	aagaccaagcagacagatgca	294	80	++++	11
273	aaagaccaagcagacagatgc	293	60	+++	12
363	aagtttcaagaattcagccct	383	66	+++	13
370	aagaattcagccctaacctct	390	50	+++	14
373	aattcagccctaacctctggg	393	50	+++	15
324	aactgtgccaaccagaccaa	344	90	++++	16
440	aaatgggtctttggagggcct	460	80	++++	17
501	aagatcctcatgaaagttgga	521	50	+++	18
513	aaagttggacaagatgcaagt	533	50	+++	19
491	aagagccatgaagatcctcat	511	50	+++	20
514	aagttggacaagatgcaagtt	534	66	+++	21
523	aagatgcaagttctgctggat	543	66	+++	22
530	aagttctgctggatcaaccag	550	50	+++	23
545	aaccaggaataaagatccaac	565	35	++	24
555	aaagatccaacaagacgtcca	575	40	++	25
556	aagatccaacaagacgtccag	576	60	+++	26
563	aacaagacgtccagaactaga	583	60	+++	27

566	aagacgtccagaactagaagc	586	70	+++	28
593	aaatggaagaagttcgacaac	613	75	++++	29
577	aactagaagctggtacaaatg	597	66	+++	30
594	aatggaagaagttcgacaaca	614	35	++	31
583	aagctggtacaaatggaagaa	603	50	+++	32
611	aacaagtccctttgtaaaacc	631	70	++++	33
599	aagaagttcgacaacaagtcc	619	70	++++	34
602	aagttcgacaacaagtccctt	622	80	++++	35
626	aaaaccaaattccaggttctag	646	50	+++	36
627	aaaccaaattccaggttctagc	647	25	+	37
628	aaccaaattccaggttctagca	648	30	++	38
632	aatccaggttctagcacaga	652	60	+++	39
633	aatccaggttctagcacagac	653	40	++	40
678	aacaacatcctcggttccgaa	698	30	++	41
681	aacatcctcggttccgaagtg	701	20	+	42
697	aagtggccttatttgcaggga	717	30	++	43
Additional Ephrin B2 RNAi probes described in the specification					
GCAGACAGAUGCACUAUUAUU					ephrin B2 264
CUGCGAUUUCCAAUUCGAUUU					ephrin B2 63
GGACUGGUACUAUACCCACUU					ephrin B2 137

Example 10. EphB4 antibodies inhibit tumor growth

Figure 57 shows results on comparison of EphB4 monoclonal antibodies by G250 and in Pull-down assay.

- 5 Figure 58 shows that EphB4 antibodies, in the presence of matrigel and growth factors, inhibit the *in vivo* tumor growth of SCC15 cells.

10 BaIbC nude mice were injected subcutaneously with 2.5×10^6 viable tumor cells SCC15 is a head and neck squamous cell carcinoma line. Tumors were initiated in nu/nu mice by injecting $2.5-5 \times 10^6$ cells premixed with matrigel and Growth factors, and Ab's subcutaneously to initiate tumor xenografts. Mice were opened 14 days after injections. SCC15 is a head and neck squamous cell carcinoma line, B16 is a melanoma cell line, and MCF-7 is a breast carcinoma line. The responses of tumors to these treatments were compared to control treated mice, which receive PBS injections. Animals were observed daily for tumor growth and subcutaneous tumors were measured using a caliper every 2 days.

Antibodies #1 and #23 showed significant regression of SCC15 tumor size compared to control, especially with no additional growth factor added.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

5 Angiogenesis was assessed by CD-31 immunohistochemistry. Tumor tissue sections from treated and untreated mice were stained for CD31. Apoptosis was assessed by immunohistochemical TUNNEL, and proliferation by BrdU assay. Following surgical removal, tumors were immediately sliced into 2 mm serial sections and embedded in paraffin using standard procedures. Paraffin embedded tissue were sectioned at 5 μ m, the wax
10 removed and the tissue rehydrated. The rehydrated tissues were microwave irradiated in antigen retrieval solution. Slides were rinsed in PBS, and TUNNEL reaction mixture (Terminal deoxynucleotidyl transferase and fluorescein labeled nucleotide solution), and BrdU were added in a humidity chamber completely shielded from light. The TUNNEL and BrdU reaction mixture were then removed, slides were rinsed and anti-fluorescein antibody
15 conjugated with horseradish peroxidase was added. After incubation and rinsing, 3, 3'-diaminobenzidine was added. Masson's Trichrome and Hematoxylin and Eosin were also used to stain the slides to visualize morphology. Masson's Trichrome allows to visualize necrosis and fibrosis. The tumor gets blood support from tumor/skin, muscle boundary. As tumor grows, inner regions get depleted of nutrients. This leads to necrosis (cell death),
20 preferably at the tumor center. After cells die, (tumor) tissue gets replaced with fibroblastic tissue. Slides were visualized under 20-fold magnification with digital images acquired. A different morphology was obtained on SCC tumors with each antibody administered. Ab #1 showed an increase in necrosis and fibrosis but not apoptosis. Ab #23 showed an increase in apoptosis, necrosis and fibrosis and a decrease in vessel infiltration. Ab #35 showed an
25 increase in necrosis and fibrosis, and a small increase in apoptosis and a decrease in vessel infiltration. Ab #79 showed a large increase in apoptosis, and necrosis and fibrosis. Ab #91 showed no change in apoptosis but an increase in proliferation. And Ab #138 showed an increase in apoptosis, necrosis, fibrosis and a decrease in proliferation and vessel infiltration. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic
30 response. Tumors treated with EphB4 antibodies displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 60 shows that systemic administration of antibodies on xenografts leads to tumor regression in SCC15 tumor xenografts.

Alternate day treatment with EphB4 monoclonal antibody or an equal volume of PBS as control were initiated on day 4, after the tumors have established, and continued for 14 days. Systemic administration was administered either IP or SC with no significant difference. All the experiments were carried out in a double-blind manner to eliminate investigator bias. Mice were sacrificed at the conclusion of the two week treatment period. Tumors were harvested immediately postmortem and fixed and processed for immunohistochemistry. EphB4 antibodies 40 mg per kg body weight were administered. Treatment with EphB4 antibody significantly inhibited human SCC tumor growth compared with control-treated mice ($p < 0.05$). Treatment with EphB4 antibody significantly inhibited tumor weight compared with control-treated mice ($p < 0.05$).

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

WE CLAIM:

1. An isolated soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide.
- 5 2. The soluble polypeptide of claim 1, comprising a globular domain of an EphB4 protein.
3. The soluble polypeptide of claim 1, comprising a sequence at least 90% identical to residues 1-522 of the amino acid sequence defined by Figure 65.
4. The soluble polypeptide of claim 1, comprising a sequence at least 90% identical to
10 residues 1-412 of the amino acid sequence defined by Figure 65.
5. The soluble polypeptide of claim 1, comprising a sequence at least 90% identical to residues 1-312 of the amino acid sequence defined by Figure 65.
6. The soluble polypeptide of claim 1, comprising a sequence defined by Figure 1 or 2.
7. The soluble polypeptide of claim 1, wherein the soluble polypeptide inhibits the
15 interaction between Ephrin B2 and EphB4.
8. The soluble polypeptide of claim 1, wherein the soluble polypeptide inhibits clustering of Ephrin B2 or EphB4.
9. The soluble polypeptide of claim 1, wherein the soluble polypeptide inhibits phosphorylation of Ephrin B2 or EphB4.
- 20 10. The soluble polypeptide of claim 1, wherein the soluble polypeptide is a fusion protein.
11. The soluble polypeptide of claim 1, wherein the soluble polypeptide comprises one or more modified amino acid residues.
12. An isolated soluble polypeptide comprising an amino acid sequence of an extracellular
25 domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.
13. The soluble polypeptide of claim 12, comprising residues 1-225 of the amino acid sequence defined by Figure 66.
14. The soluble polypeptide of claim 12, comprising a sequence defined by Figure 3.
- 30 15. The soluble polypeptide of claim 12, wherein the soluble polypeptide inhibits the interaction between Ephrin B2 and EphB4.
16. The soluble polypeptide of claim 12, wherein the soluble polypeptide inhibits clustering of Ephrin B2 or EphB4.

17. The soluble polypeptide of claim 12, wherein the soluble polypeptide inhibits phosphorylation of Ephrin B2 or EphB4.
18. The soluble polypeptide of claim 12, wherein the soluble polypeptide is a fusion protein.
- 5 19. The soluble polypeptide of claim 12, wherein the soluble polypeptide comprises one or more modified amino acid residues.
20. A pharmaceutical composition comprising the soluble polypeptide of claim 1, and a pharmaceutically acceptable carrier.
21. A pharmaceutical composition comprising the soluble polypeptide of claim 12, and a
10 pharmaceutically acceptable carrier.
22. A cosmetic composition comprising the soluble polypeptide of claim 1, and a pharmaceutically acceptable carrier.
23. A cosmetic composition comprising the soluble polypeptide of claim 12, and a pharmaceutically acceptable carrier.
- 15 23. A diagnostic kit comprising the soluble polypeptide of claim 1 and a pharmaceutically acceptable carrier.
24. A diagnostic kit comprising the soluble polypeptide of claim 12 and a carrier.
25. An antagonist antibody selected from the group consisting of:

20 (a) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(b) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.
26. The antagonist antibody of claim 25, wherein the antagonist antibody inhibits the interaction between Ephrin B2 and EphB4.
- 25 27. The antagonist antibody of claim 25, wherein the antagonist antibody inhibits clustering of Ephrin B2 or EphB4.
28. The antagonist antibody of claim 25, wherein the antagonist antibody inhibits phosphorylation of Ephrin B2 or EphB4.
29. The antagonist antibody of claim 25, wherein the antagonist antibody is a monoclonal
30 antibody.
30. The antagonist antibody of claim 25, wherein the antagonist antibody is a polyclonal antibody.

31. A pharmaceutical composition comprising the antagonist antibody of claim 25, and a pharmaceutically acceptable carrier.
32. A cosmetic composition comprising the antagonist antibody of claim 25, and a pharmaceutically acceptable carrier.
- 5 33. A diagnostic kit comprising the antagonist antibody of claim 25, and a carrier.
34. A method of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell, comprising contacting the cell with an effective amount of a soluble polypeptide of claim 1.
- 10 35. A method of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell, comprising contacting the cell with an effective amount of a soluble polypeptide of claim 12.
36. A method of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell, comprising contacting the cell with an effective amount of a soluble polypeptide of claim 25.
- 15 37. A method of reducing the growth rate of a tumor, comprising administering an amount of a polypeptide agent sufficient to reduce the growth rate of the tumor, wherein the polypeptide agent is selected from the group consisting of:
- 20 (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;
- (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.
- 25 (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and
- (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.
38. The method of claim 37, wherein the tumor comprises cells expressing a higher level of EphB4 and/or EphrinB2 than noncancerous cells of a comparable tissue.
- 30 39. A method for treating a patient suffering from a cancer, comprising administering to the patient a polypeptide agent selected from the group consisting of:
- (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

(b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

40. The method of claim 39, wherein the cancer comprises cancer cells expressing EphrinB2 and/or EphB4 at a higher level than noncancerous cells of a comparable tissue.

41. The method of claim 39, wherein the cancer is metastatic cancer.

42. The method of claim 39, wherein the tumor is selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.

43. The method of claim 39, wherein the cancer is an angiogenesis-dependent cancer.

44. The method of claim 39, wherein the cancer is an angiogenesis-independent cancer.

45. The method of claim 39, wherein the polypeptide agent inhibits the interaction between Ephrin B2 and EphB4.

46. The method of claim 39, wherein the polypeptide agent inhibits clustering of Ephrin B2 or EphB4.

47. The method of claim 39, wherein the polypeptide agent inhibits phosphorylation of Ephrin B2 or EphB4.

48. The method of claim 39, wherein the polypeptide agent is formulated with a pharmaceutically acceptable carrier.

49. The method of claim 39, further including administering at least one additional anti-cancer chemotherapeutic agent that inhibits cancer cells in an additive or synergistic manner with the polypeptide agent.

50. A method of inhibiting angiogenesis, comprising contacting a cell an amount of a polypeptide agent sufficient to inhibit angiogenesis, wherein the polypeptide agent is selected from the group consisting of:

(a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

(b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

51. The method of claim 50, wherein the cell expresses EphB4 or Ephrin B2.

52. A method for treating a patient suffering from an angiogenesis-associated disease, comprising administering to the patient a polypeptide agent selected from the group consisting of:

(a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

(b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

53. The method of claim 52, wherein the soluble polypeptide is formulated with a pharmaceutically acceptable carrier.

54. The method of claim 52, wherein the angiogenesis-associated disease is selected from the group consisting of angiogenesis-dependent cancer, benign tumors, inflammatory disorders, chronic articular rheumatism and psoriasis, ocular angiogenic diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, wound granulation, wound healing, telangiectasia psoriasis scleroderma, pyogenic granuloma, coronary collaterals, ischemic limb angiogenesis, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, and hematopoiesis.

55. The method of claim 52, further including administering at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the soluble polypeptide.

56. Use of a polypeptide agent in the manufacture of medicament for the treatment of cancer, wherein the polypeptide agent is selected from the group consisting of:
- (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;
 - (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.
 - (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and
 - (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.
57. Use of a polypeptide agent in the manufacture of medicament for the treatment of an angiogenesis-associated disease, wherein the polypeptide agent is selected from the group consisting of:
- (a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;
 - (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.
 - (c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and
 - (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.
58. A method for treating a patient suffering from a cancer, comprising:
- (a) identifying in the patient a tumor having a plurality of cancer cells that express EphB4 and/or EphrinB2; and
 - (b) administering to the patient a polypeptide agent selected from the group consisting of:
 - (i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

(ii) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(iii) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(iv) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

59. A method for treating a patient suffering from a cancer, comprising:

(a) identifying in the patient a tumor having a plurality of cancer cells having a gene amplification of the EphB4 and/or EphrinB2 gene; and

(b) administering to the patient a polypeptide agent selected from the group consisting of:

(i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

(ii) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(iii) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

(iv) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

60. A method for identifying a tumor that is suitable for treatment with an EphrinB2 or EphB4 antagonist, the method comprising detecting in the tumor cell one or more of the following characteristics:

(a) expression of EphB4 protein and/or mRNA;

(b) expression of EphrinB2 protein and/or mRNA;

(c) gene amplification of the EphB4 gene; and

(d) gene amplification of the EphrinB2 gene,

wherein a tumor cell having one or more of characteristics (a)-(d) is suitable for treatment with an EphrinB2 or EphB4 antagonist.

61. The method of claim 60, wherein the EphrinB2 or EphB4 antagonist is selected from the group consisting of:

(a) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide;

5 (b) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

(c) an antibody which binds to an extracellular domain of an EphB4 protein and inhibits an activity of the EphB4; and

10 (d) an antibody which binds to an extracellular domain of an Ephrin B2 protein and inhibits an activity of the Ephrin B2.

Figure 1. Amino acid sequence of the B4ECv3 protein

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSG
LDEEQHSVRTYEVCVQRAPGQAHWLRTGWVPRRGAVHVIATLRETM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNKTLRLGPLSKAGFYLAFOQOGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVPVAGSCVVDVAVPAGPSP
SLYCREDGQWAEQPVTCSCAPGFEEAEGNTKCRACAQGTFFKPLSGE
GSCQPCPANSHTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLES GGREDLTYALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRILQIEGKPIPNPLLGLDSTRTG
HHHHHH

Figure 2. Amino acid sequence of the B4ECv3NT protein

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSG
LDEEQHSVRTYEVCEVQRAPGOAHWLRTGWVPRRGAVHVYATLRFTM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNKTLRLGPLSKAGFYLAFOQDQGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVPVAGSCVVDVAVPAPGPSP
SLYCREDGQWAEQPVGTGCSCAPGFEEAEGNTKCRACAQGTFKPLSGE
GSCQPCPANSHTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLES GGREDLT YALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTS ENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRAILQISSTVAAARV

Figure 3. Amino acid sequence of the B2EC protein

MAVRRDSVWKYCWGVLMLCRTAISKSIVLEPIYWNSSNSKFLP
GQGLVLYPQIGDKLDIICPKVDSKTVGQYEYYKVYMVDKDQADR
CTIKKENTPLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDY
YIISTSNGLSLEGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRN
KDPTRRPELEAGTNGRSSTTSPEVKPNPGSSTDGNSAGHSGNNI
LGSEVGSHHHHHH

Figure 4. Amino acid sequence of the B4ECv3-FC protein

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEEL
SGLDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVIATL
RFTMLECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPY
IKVDTVAAEHLTRKRPGAEATGKVVNKTLLRLGPLSKAGFYLAQD
QGACMALLSLHLFYKKCAQLTVNLTREPETVPRELVPVAGSCVV
DAVPAPGPPSPSLYCREDGQWAEQPVTCSCAPGFEEAEGNTKCRA
CAQGTFFKPLSGEGSCQPCPANSHTIGSAVCQCRVGYFRARTDP
RGAPCTTPPSAPRSVVSRLNGSSLHLEWSAPLES GGREDLTYALR
CRECRPGGSCAPCGDLTFDPGPRDLVEPWVVVRGLRPDFTYTFE
VTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSPSSL
SLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELR
GLKRGASYLVQVRARSEAGYGPFQGEHHSQTQLDESEGWREQDPE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL
PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDS DGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSL
SLSPGK

Figure 5. Amino acid sequence of the B2EC-FC protein

MAVRRDSVWKYCWGVLMVLCRTAISK\$IVLEPIYWNSNSKFLPGQ
GLVLYPQIGDKLDIICPKVDSKTVGQY EYK VYMVDKDQADRCTIK
KENTPLLNCAPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIIST
NGSLEGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNKDPTRRPE
LEAGTNGRSSTTSPFVKPNPGSSTDGNSAGHSGNNILGSEVDPEPK
SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHREALHNHYTQKSLSLSPGK

Fig. 6. B4EC-FC binding assay (Protein A-agarose based)

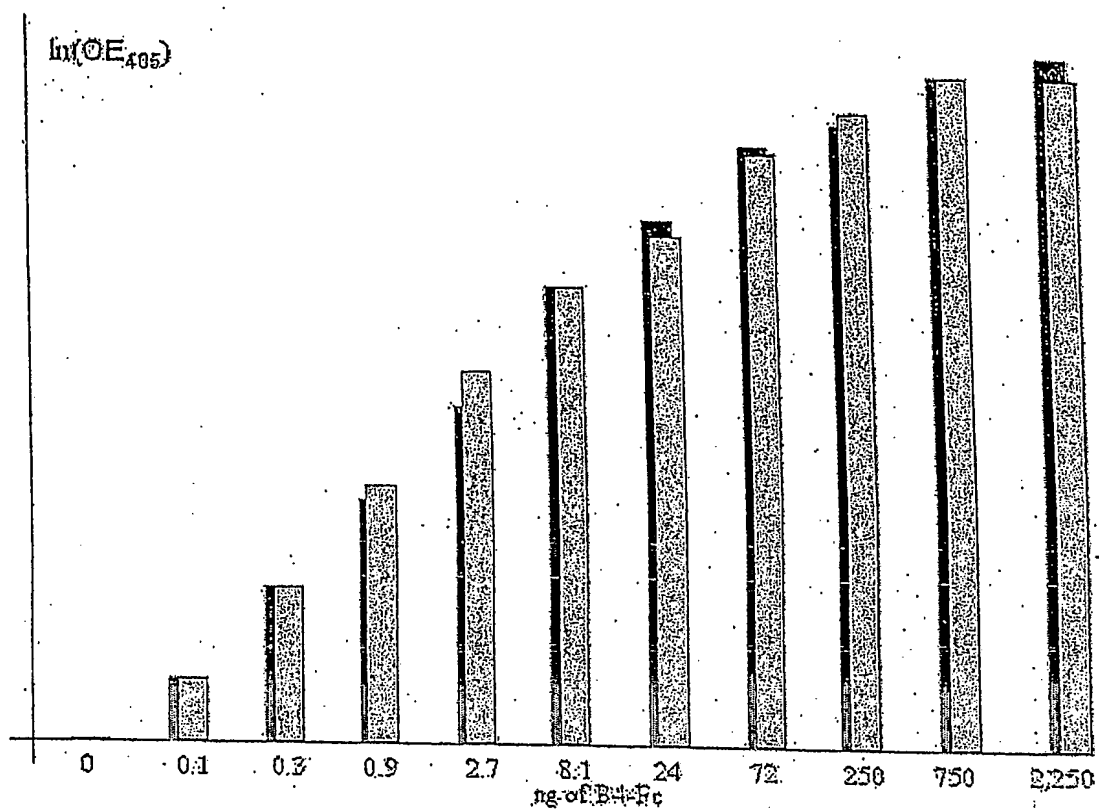


Fig. 7. B4EC-FC inhibition assay (Inhibition in solution)

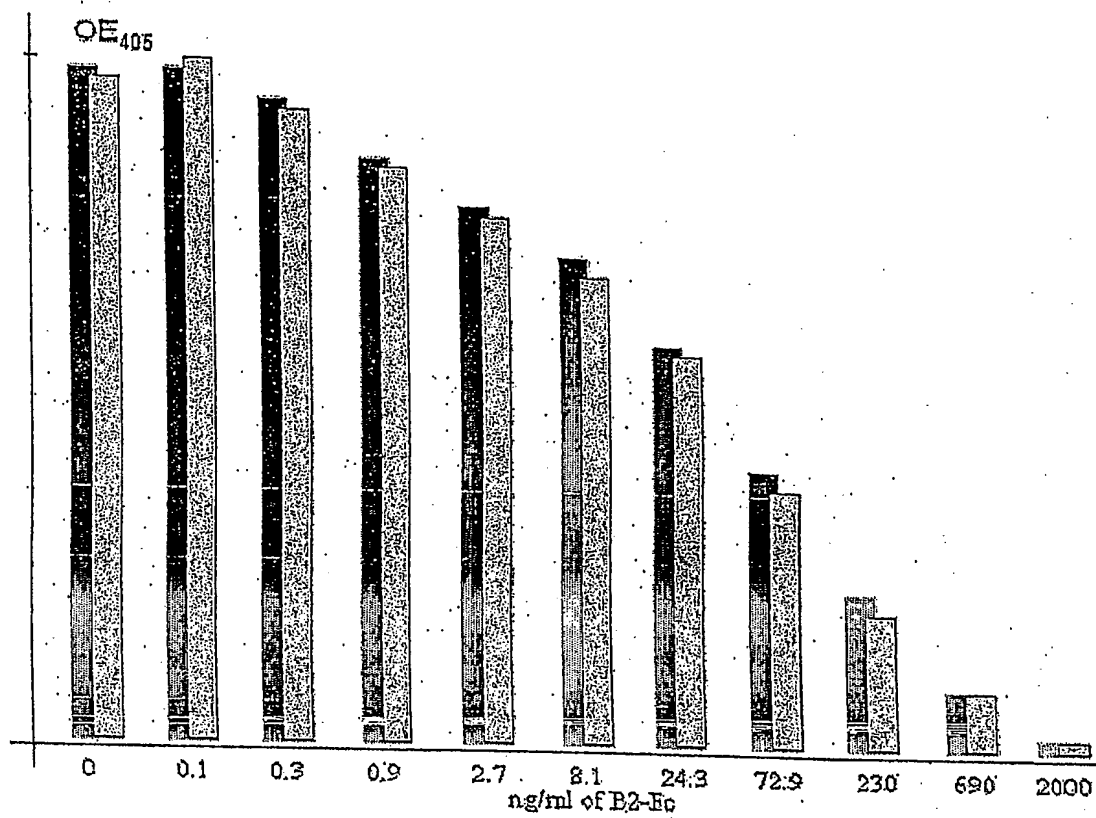


Fig. 8. B2EC-FC binding assay (Protein-A-agarose based assay)

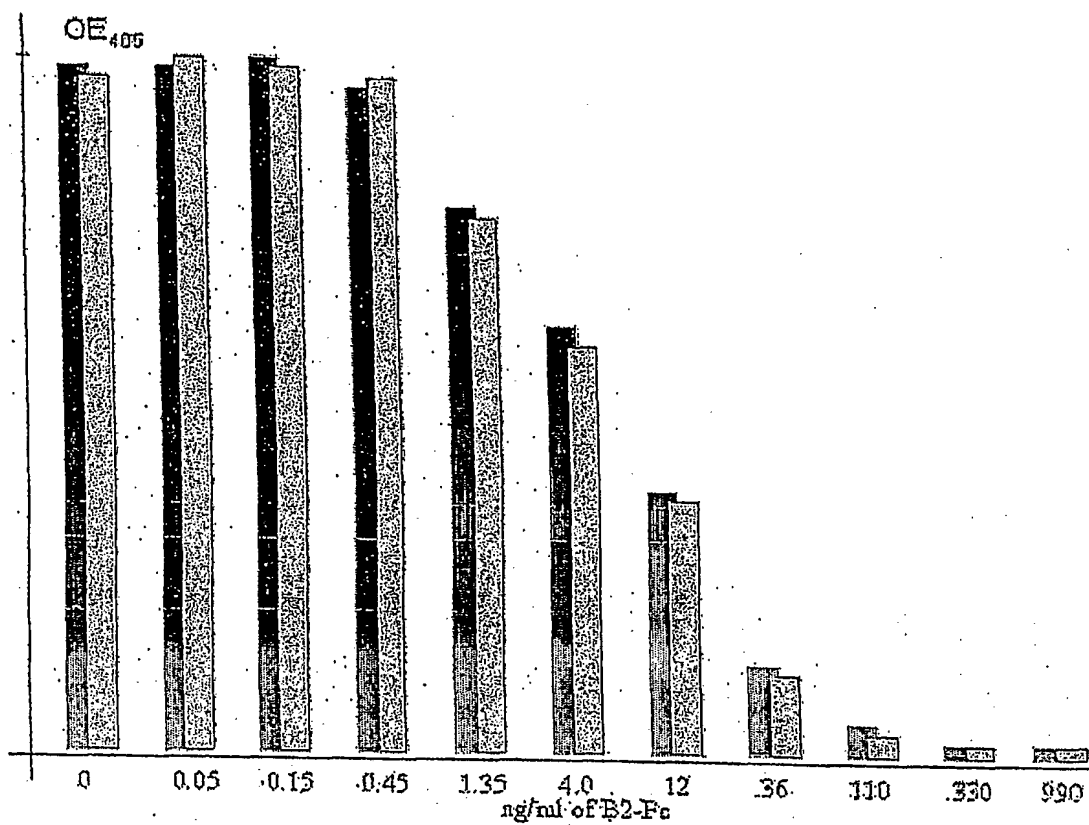


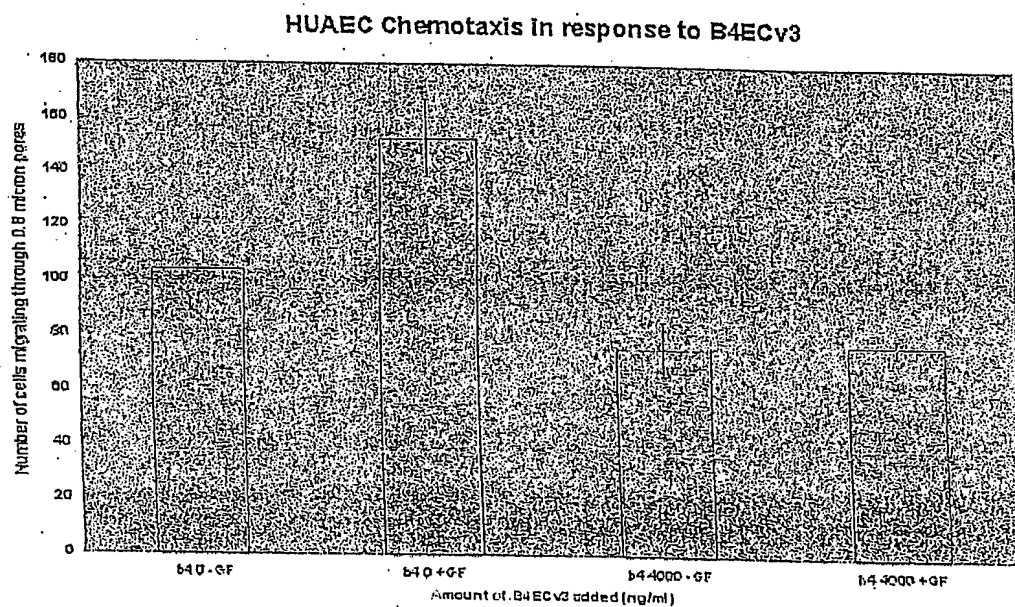
Fig. 9

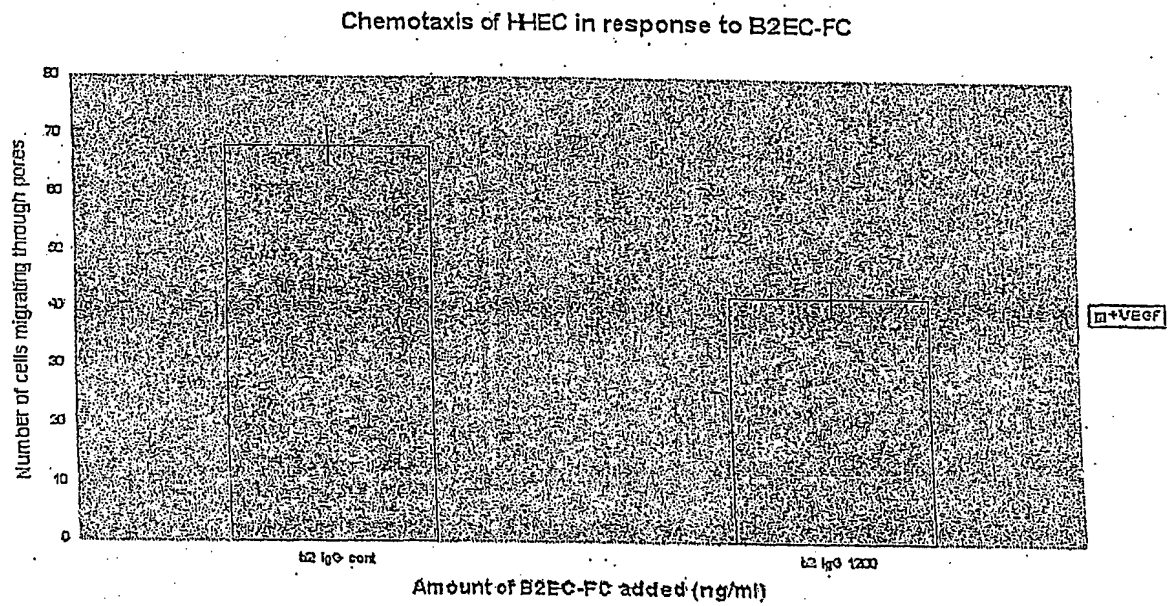
Fig. 10

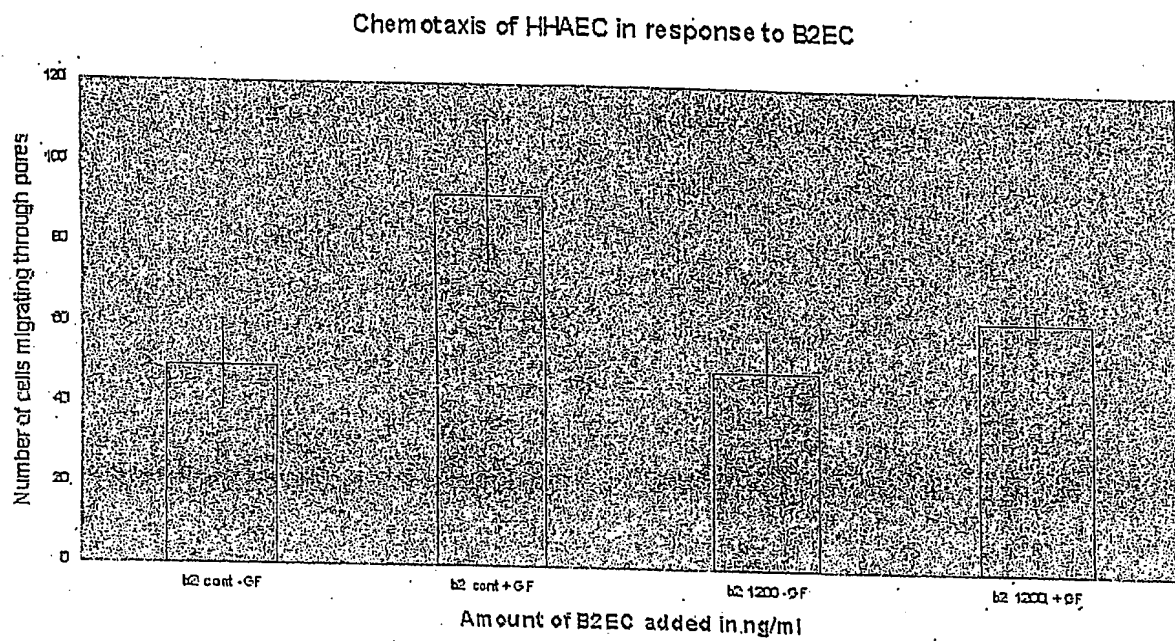
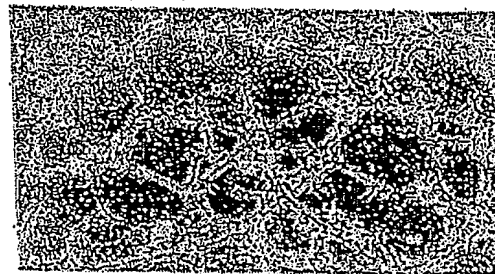
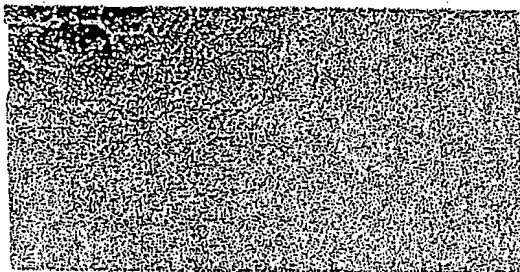
Fig. 11

Fig. 12**Effect of B4ECv3 on HUAEC Tubule Formation**B4ECv3
µg/ml

10 x magnification

20 x magnification

0



2

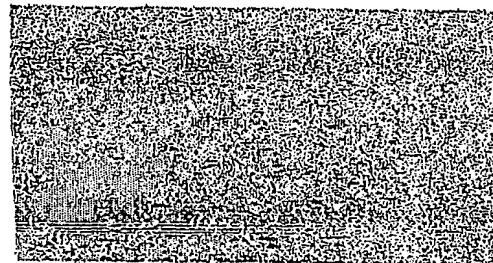
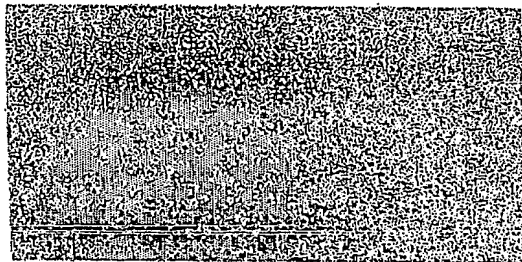
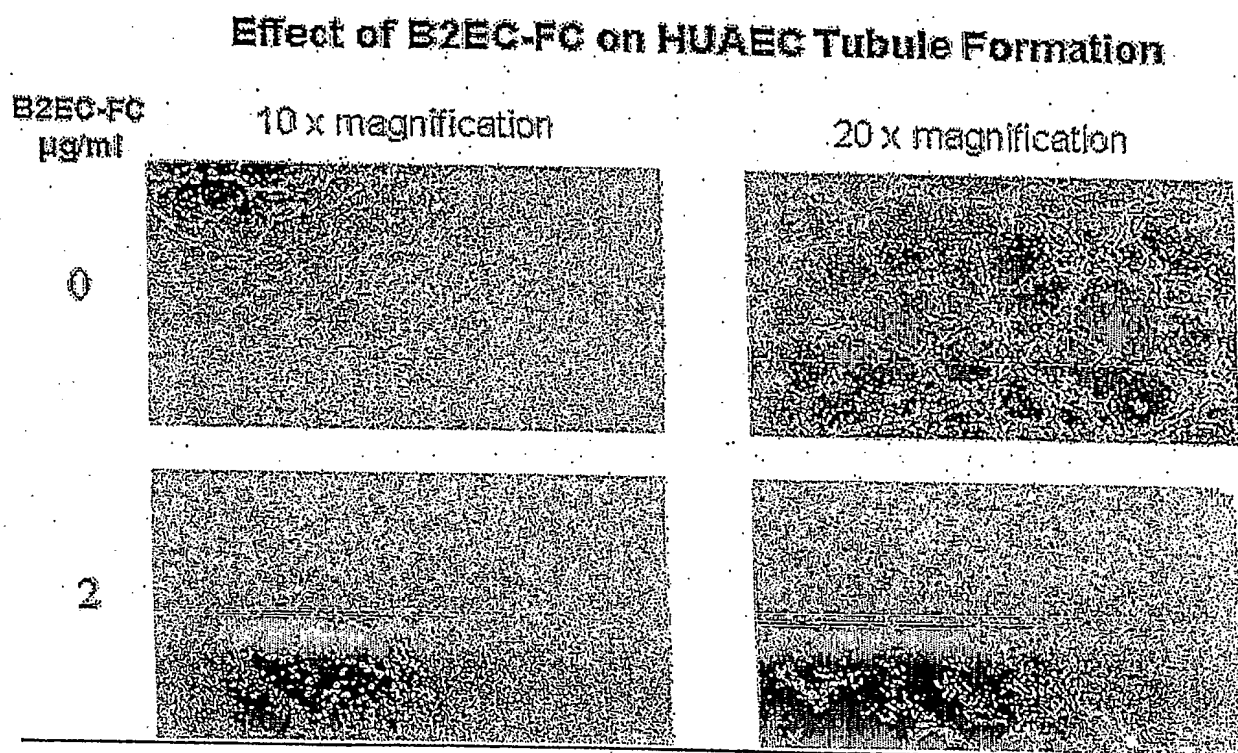


Fig. 13

hEphrin B2 constructs

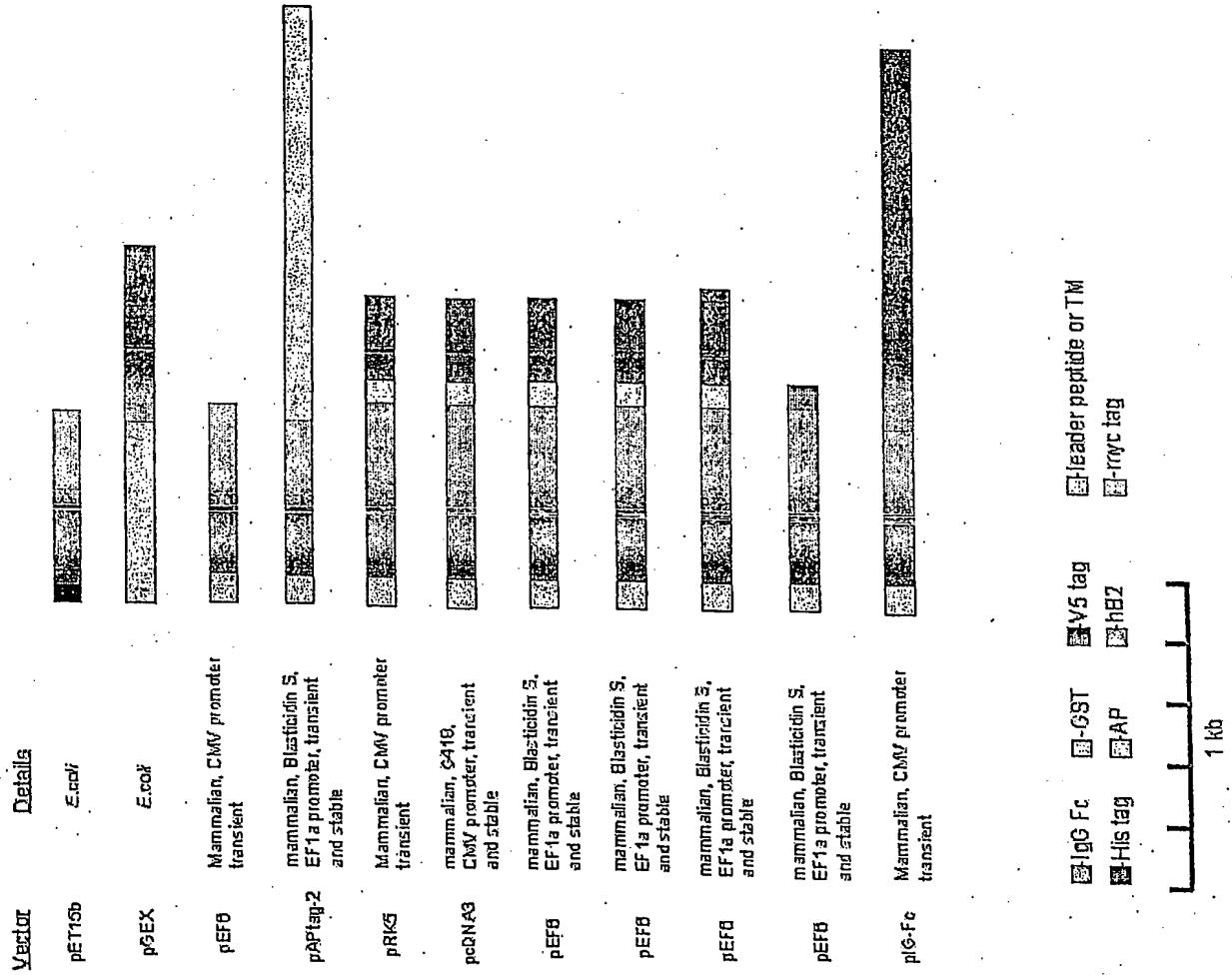
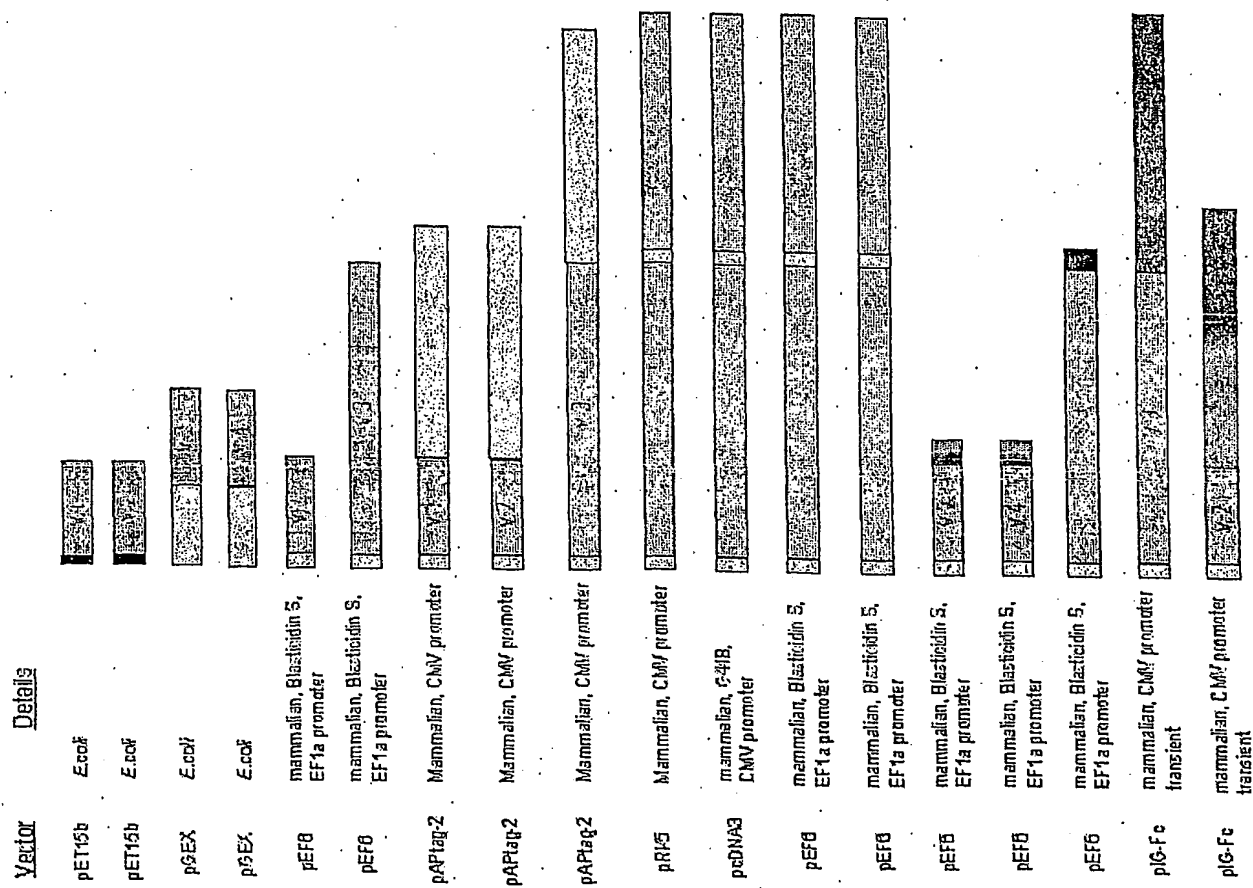


Figure 14

nprn B4 constructs







 IgG Fc
  GST
  V5 tag
  Leader peptide or TM

Figure 15

Figure 16. Domain structure of the recombinant soluble EphB4EC proteins.

Fig.1

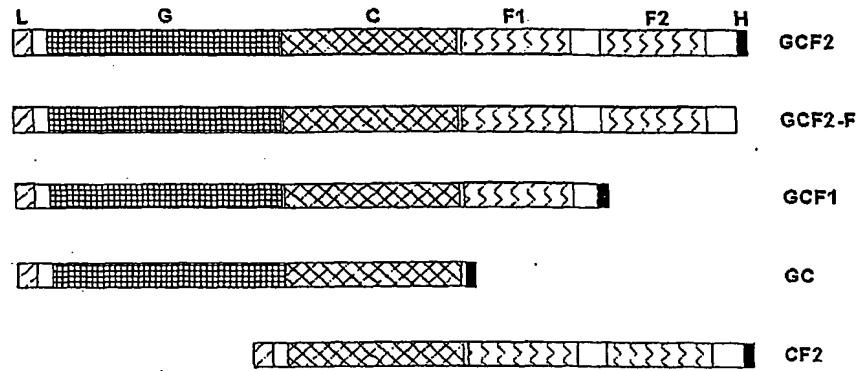


Figure 17A. Purification and ligand binding properties of the EphB4EC proteins

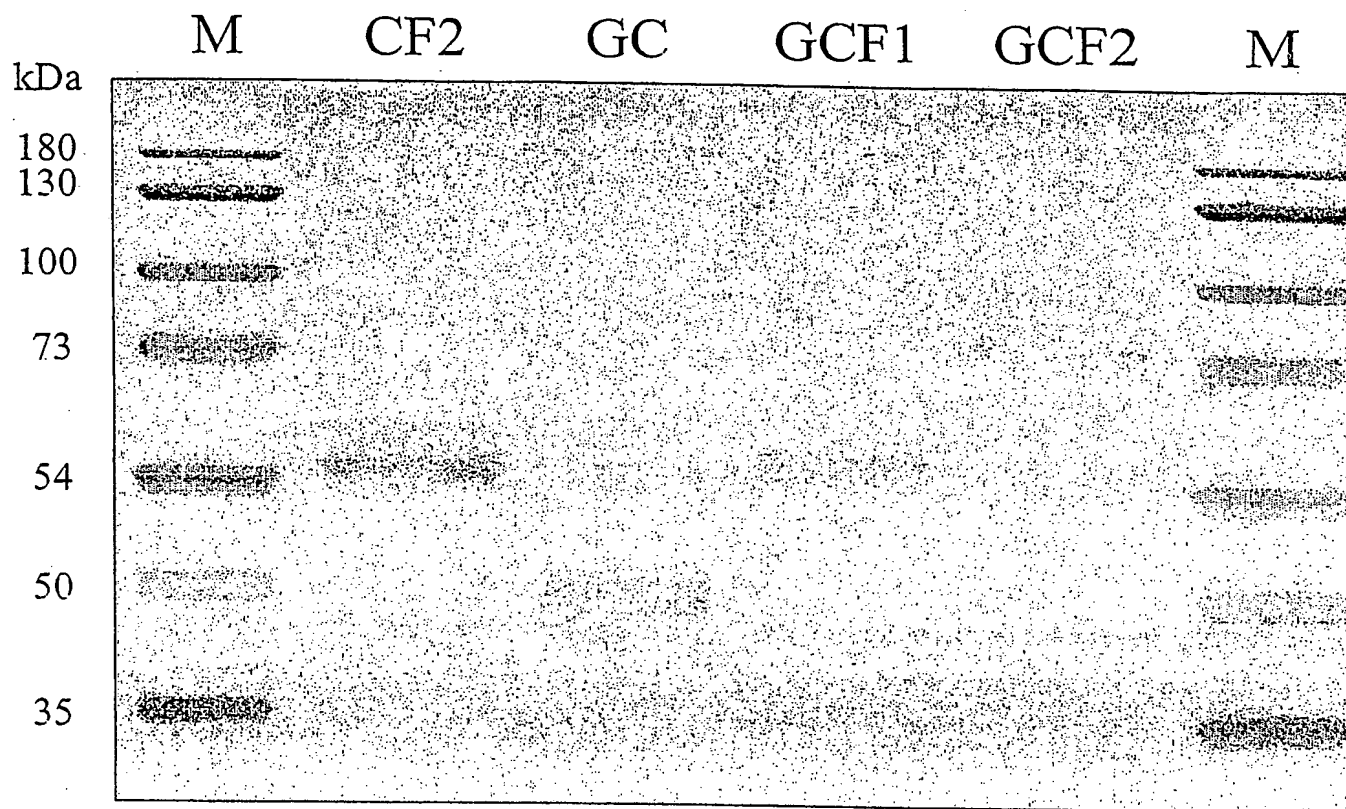


Figure 17B. Binding of Ephrin B2-AP fusion to EphB4-derived recombinant proteins immobilized on NTA-agarose beads.

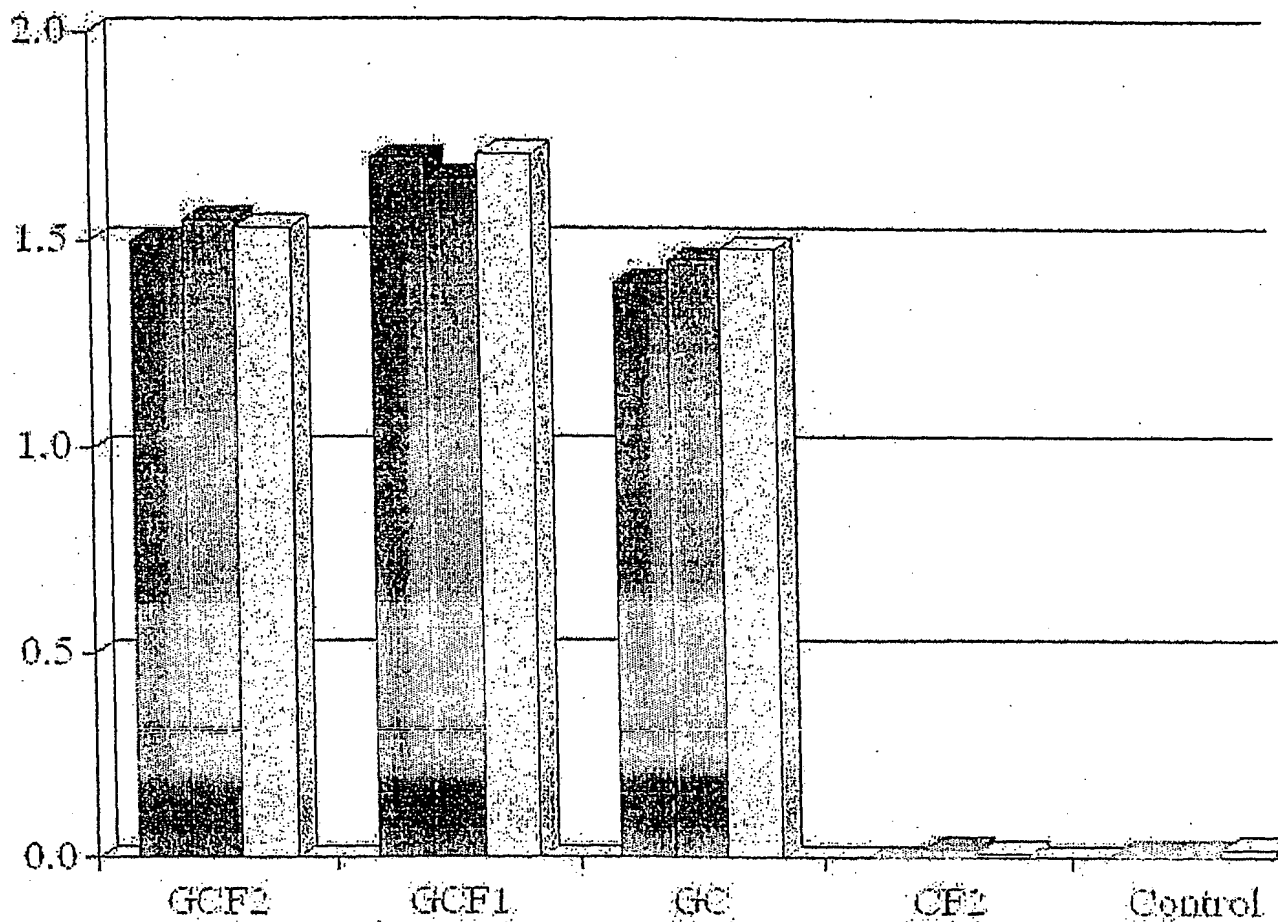
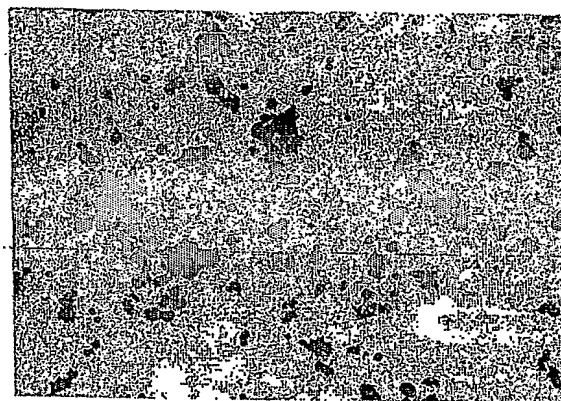
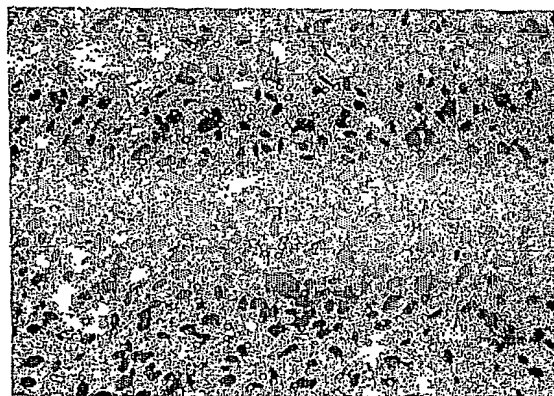
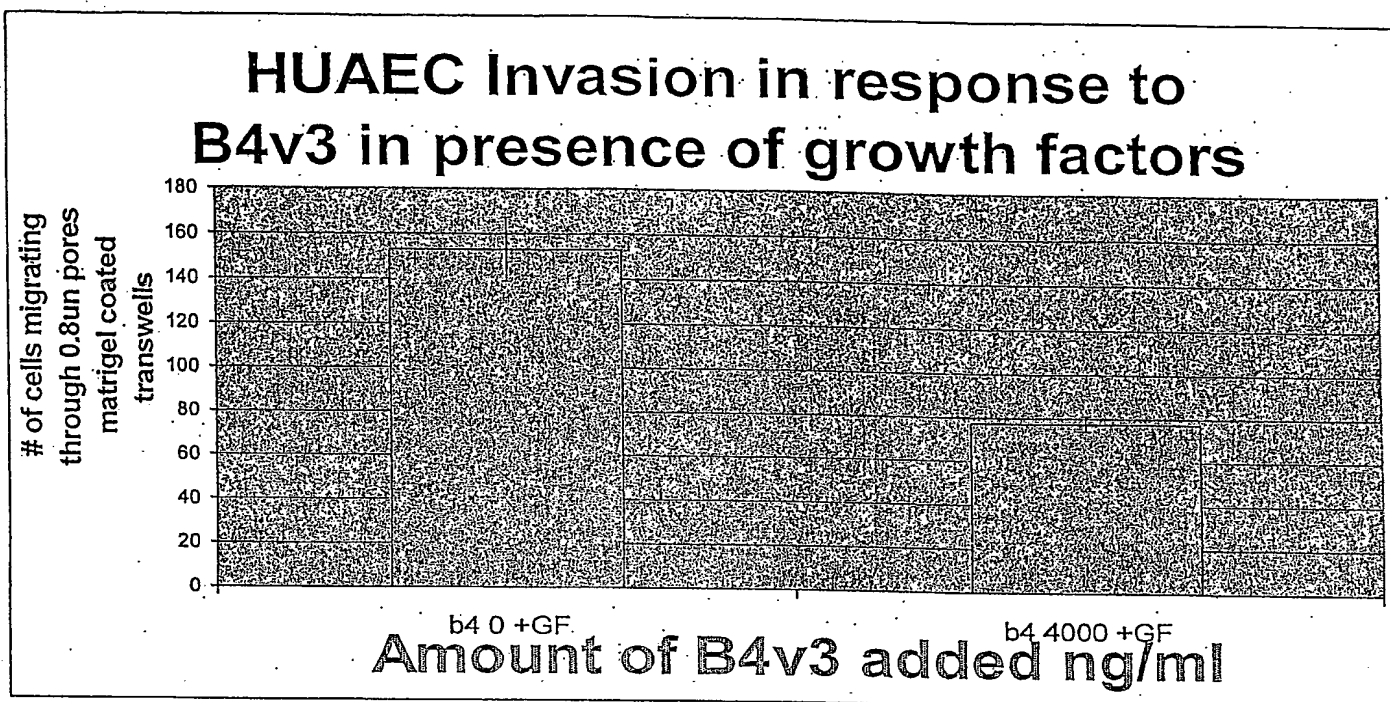


Fig. 18

19/88

B4v3 inhibits chemotaxis, In Vitro Invasion Assay

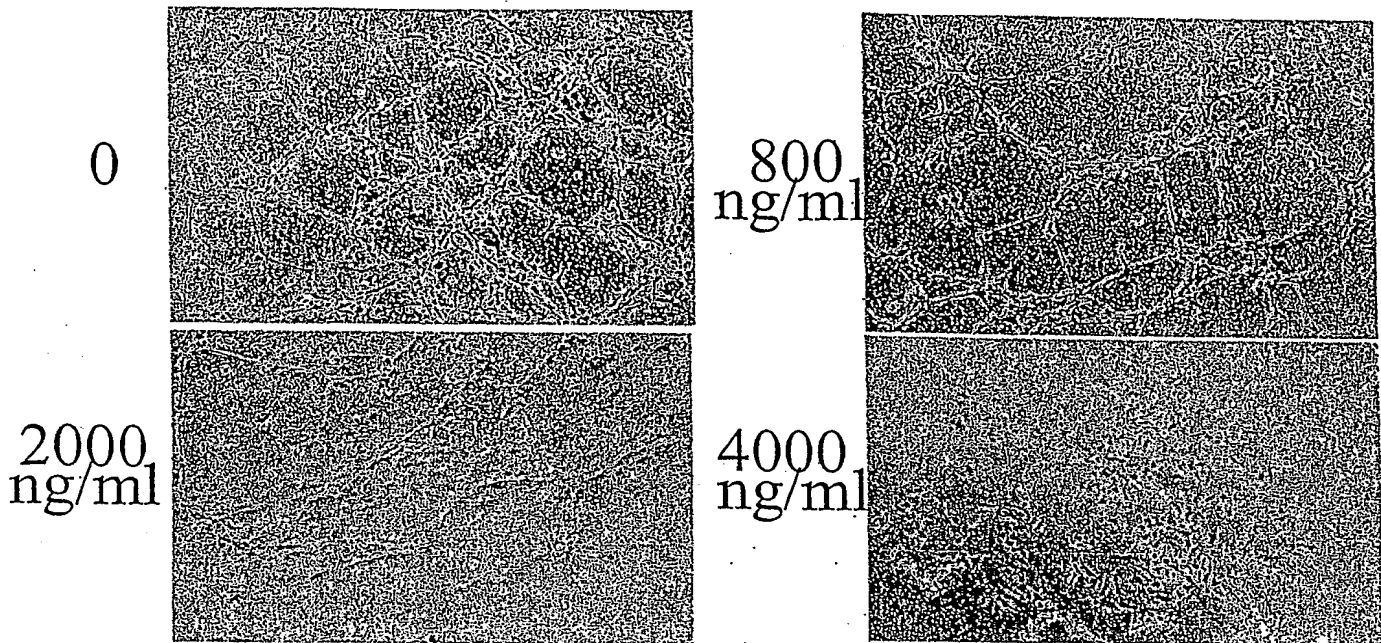


In Vitro Invasion Assay: Chemotaxis of HUAECs, measuring migration and degradation of basement membrane ability, was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24 well plates; 6.5 mm diam, 8µm pore size, 10µm thick polycarbonate membranes. The upper surfaces of the transwell were pre-coated with matrigel. The cell suspensions of HUAECs in 0.25% BSA (2×10^5 cells/ml) in 200µl of EBM were seeded in the upper chamber and the B4v3 protein was added simultaneously with stimulant (VEGF or bFGF) to the lower compartment of the chamber and their migration across a polycarbonate filter in response to 10-20 ng/ml of VEGF with or without 100nM-1µM test compound was investigated. After incubation for 4-24h at 37, The upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Triplicate. Ten random fields at 200x mag were counted and the results expressed as mean # per field

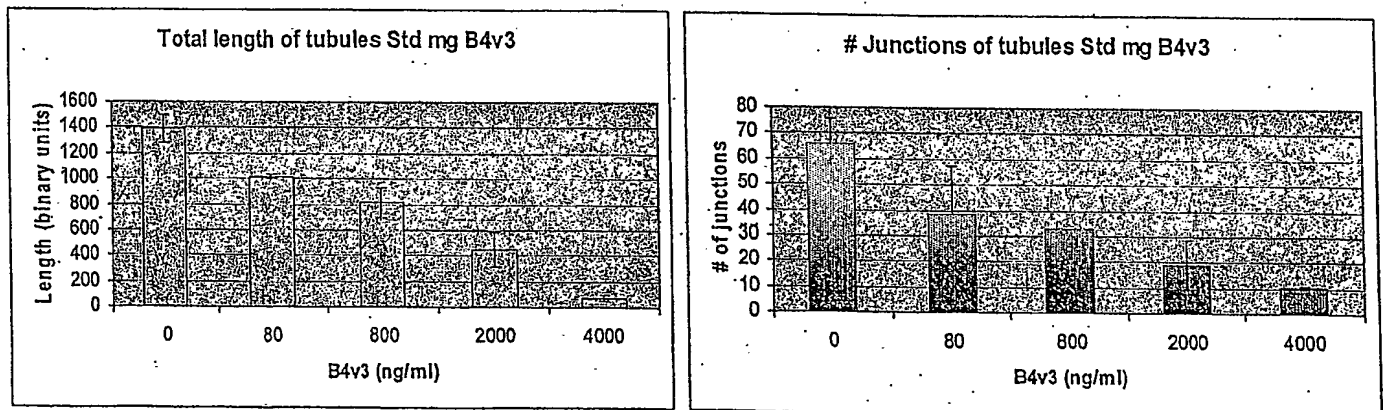
Fig. 19

20/88
B4v3 inhibits tubule formation on Matrigel.

A



B



B4v3 inhibits tubule formation on Matrigel.

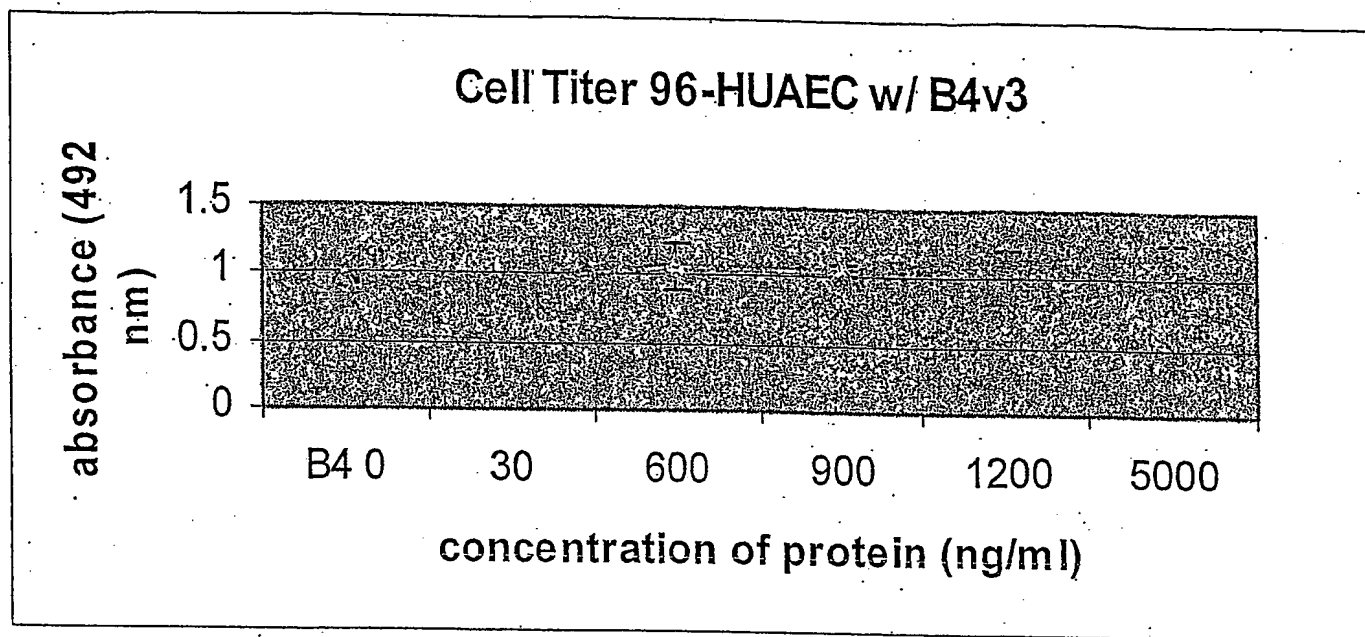
HUAEC cultures were cultured with B4v3, at 800, 2000, and 4000 ng/ml following seeding on STD matrigel in growth factor stimulated conditions, to analyze tubule formation. Cells were photographed 6h and 24h after seeding, 20X magnification, and the total length of the tubule-like network formed in the well, and # of junctions was established.

A, displays the strong inhibition of tubule formation by B4v3 in a representative experiment.

B, shows a quantitation, with AngioSys Software, of the reduction of tube-length obtained with B4v3 at increasing concentrations as well as a reduction in the number of junctions, in comparison to cells with no protein. Results are displayed as mean values \pm S.D. obtained from three independent experiments performed with duplicate wells.

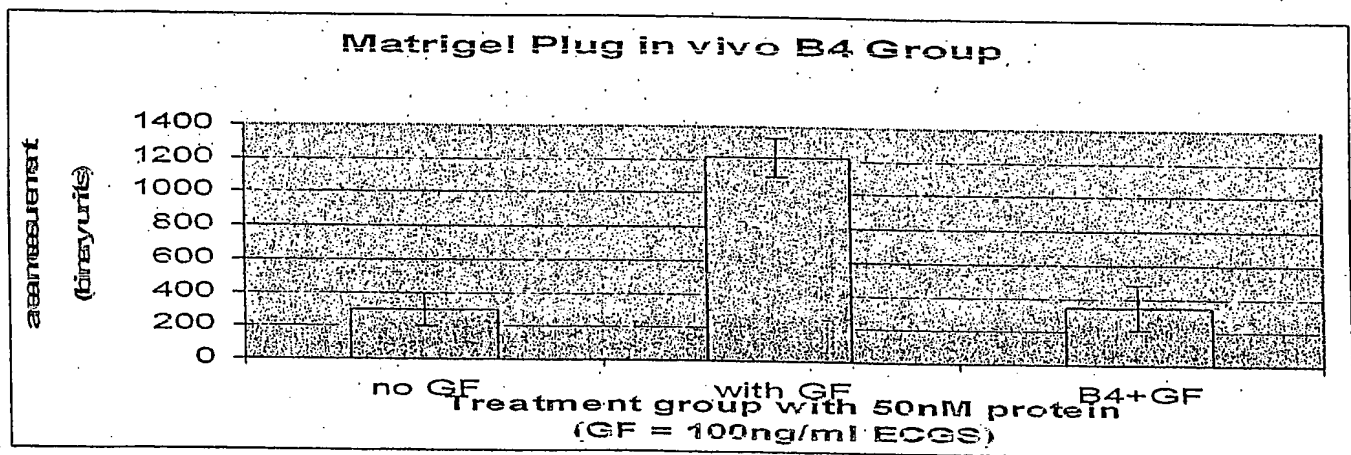
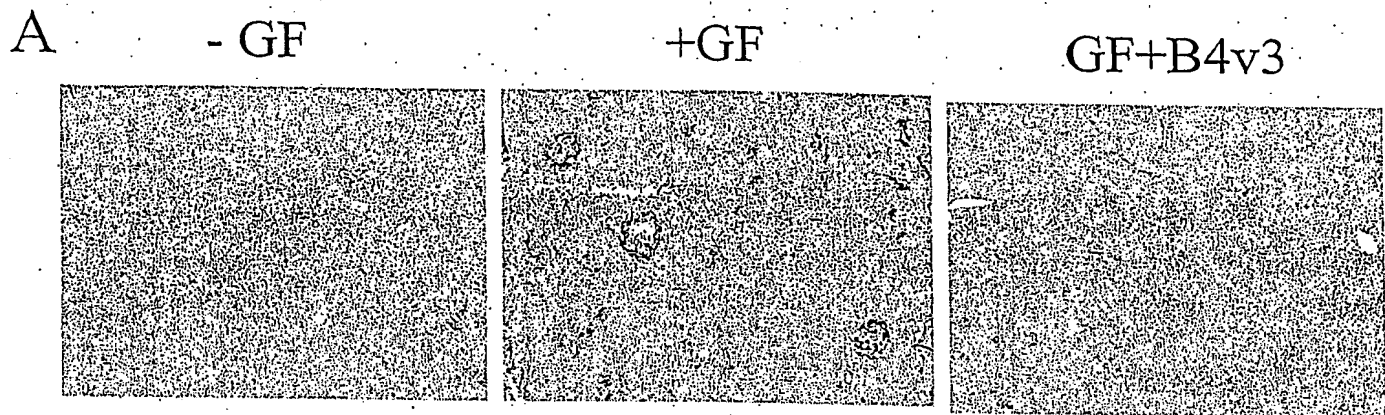
Fig. 20

21/88

**Cell viability assays:**

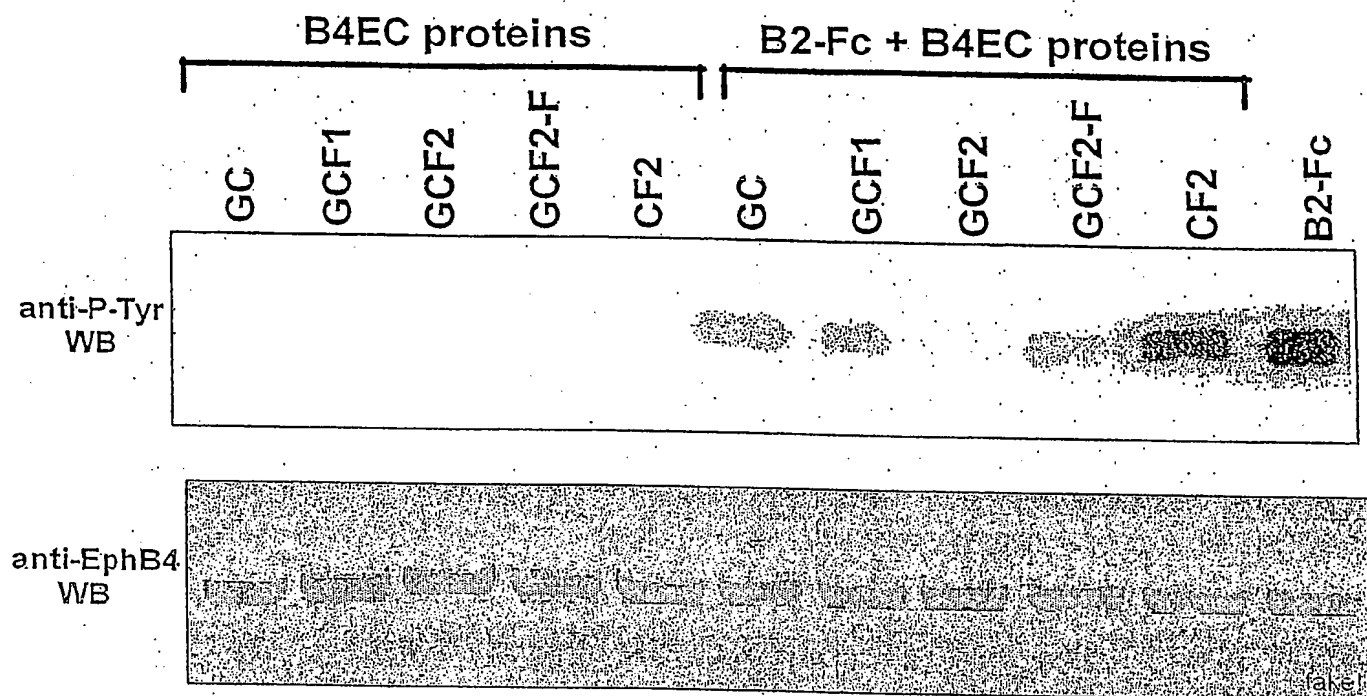
Cell viability was determined using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay according to the instructions of the manufacturer (Promega, Madison, WI, USA).

B4v3 inhibits invasion and tubule formation by endothelial cells in the Murine Matrigel assay



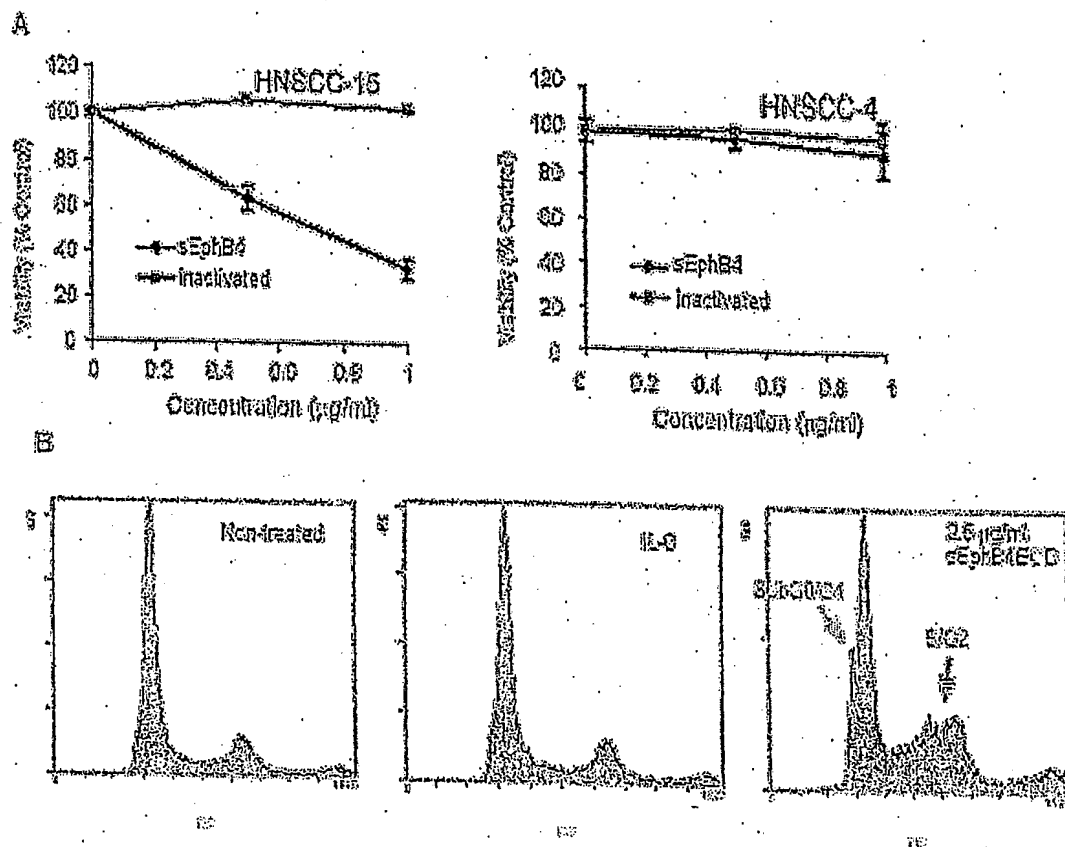
B4v3 inhibits invasion and tubule formation by endothelial cells in the murine Matrigel assay. B4v3 (50nM) was added to Matrigel solution containing ECGS (150ng/ml) and injected subcutaneously into BalbC nu/nu mice. After 6 days plugs were removed and processed in paraffin. Individual sections were either stained with hematoxylin (A) to detect total invading cells, photographed at 20X magnification or with Masson's Trichrome Top left of A B displays section of a Matrigel plug with no GF, top right of A displays section with B4IgG containing GF and lower left section contains GF, and lower right shows GF in the presence of B4v3. Significant invasion of endothelial cells is only seen in GF containing Matrigel. Top right displays an area with a high number of invaded cells induced by B4IgG, which signifies the dimeric form of B4v3. The left upper parts of the pictures correspond to the cell layers formed around the Matrigel plug from which cells invade toward the center of the plug located in the direction of the right lower corner. Total cells in sections of the Matrigel plugs were quantitated with Scion Image software. Results obtained from two experiments with duplicate plugs are displayed as mean values _ S.D.

Fig. 22



Tyrosine phosphorylation of EphB4 receptor in PC3 cells in response to stimulation with EphrinB2-F fusion in presence or absence of EphB4-derived recombinant soluble proteins.

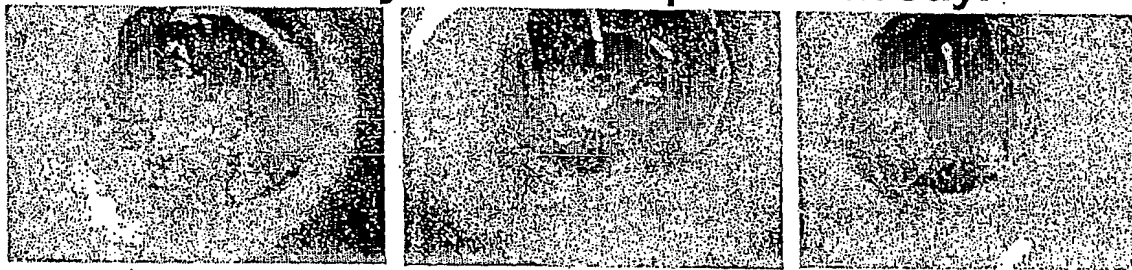
Fig. 23



Panel A: sEphB4EC effects on viability and cell cycle. **A)** 3-day cell viability assay of two HNSCC cell lines. Cells were seeded on 48-well plates at equal densities and treated with 0, 0.2 or 1 μg/ml sEphB4EC. Viability was determined on day 3 by MTT assay. Shown is the mean and SEM of triplicate samples. **B)** FACS analysis of cell cycle in HNSCC-15 cells treated as in A. It was previously determined that IL-6 had no inhibitory effect on viability. Treatment of these cells resulted in an accumulation in subG0/G1 and S/G2 phases as indicated by the arrows.

Fig. 24

B4v3 inhibits neovascular response in a murine corneal hydon micropocket assay.



+GF

B4

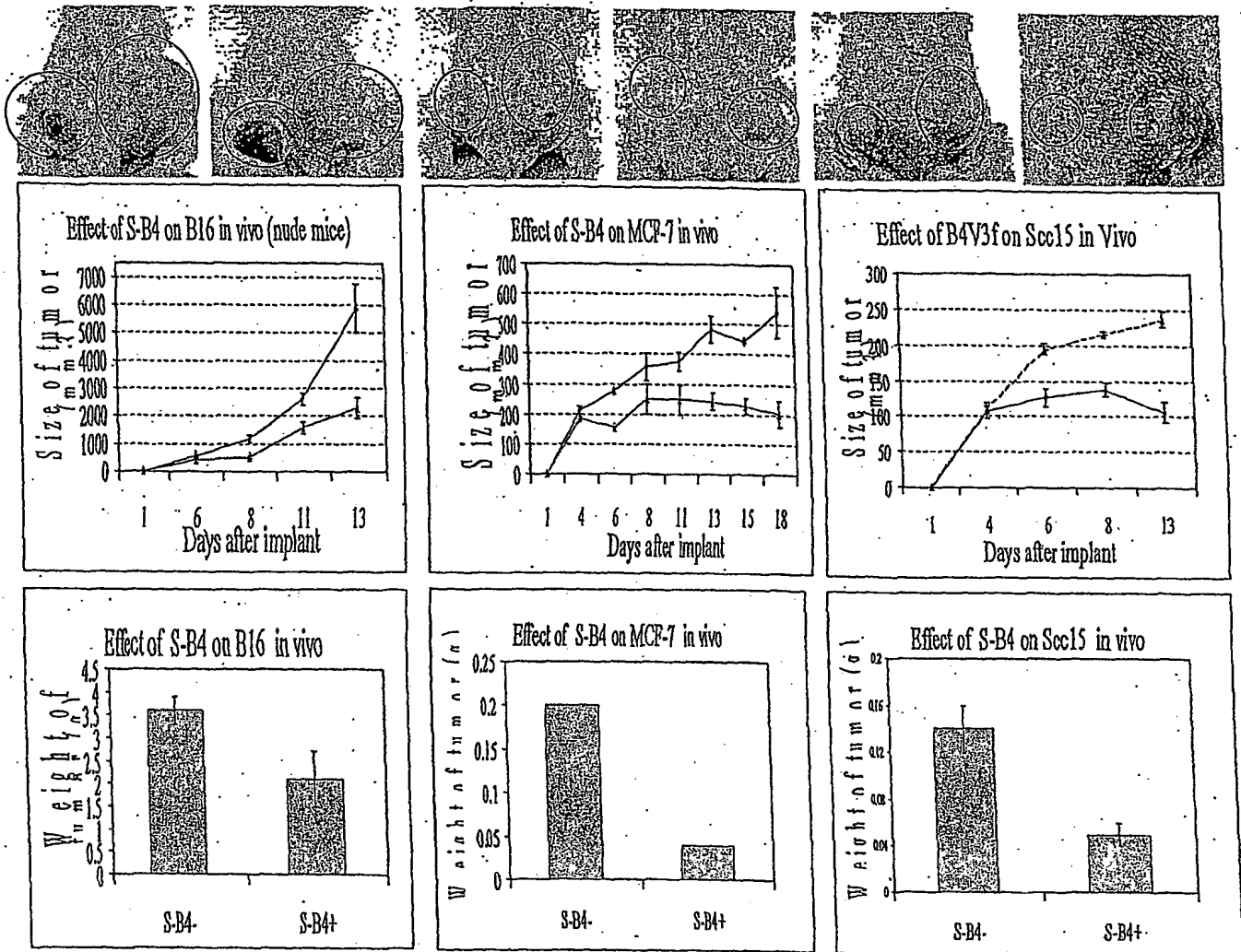
+GF

-GF

B4v3 inhibits neovascular response in a murine corneal hydon micropocket assay.

B4v3 (180ng) was added to hydon and sucralfate (45ug) with or without basic fibroblast growth factor (bFGF) (100ng) and pellets formed. The pellets were selected and inserted into a micropocket into corneas of BalbC nu/nu mice. After 3 days pellets were removed and processed in freezing compound. Only the bFGR-sucralfate pellet, top left, induced an intense neovascular response originating from the limbal vessels and reaching the pellet on day 3 after implantation. Pellets containing bFGF and sucralfate with B4v3 and Bsf, top right and bottom left respectively, did not produce an angiogenic response above background, lower right, on day 3 after implantation.

Fig. 25



SCC15, B16, and MCF-7 co-injected with sB4v3 in the presence of matrigel and growth factors, inhibits the *in vivo* tumor growth of these cells. (A) sB4v3, 40mg per kg body weight were subcutaneously co-injected with $\times 10^6$ cells in a matrigel preparation. The representative picture shows retarded tumor growth in the presence of sB4 (left flank) compared with PBS control treatment (right flank). (B) Treatment with sB4 significantly inhibited human SCC, B16, and MCF-7 tumor growth compared with control-treated mice ($p < 0.05$). (C) Treatment with sB4 significantly inhibited tumor weight compared with control-treated mice ($p < 0.05$). Data are expressed as mean \pm SEM. * $p < 0.05$

SCC15 Tumor histology

Fig. 26

Fite-

Lectin

-sB4

+sB4

-sB4

+sB4

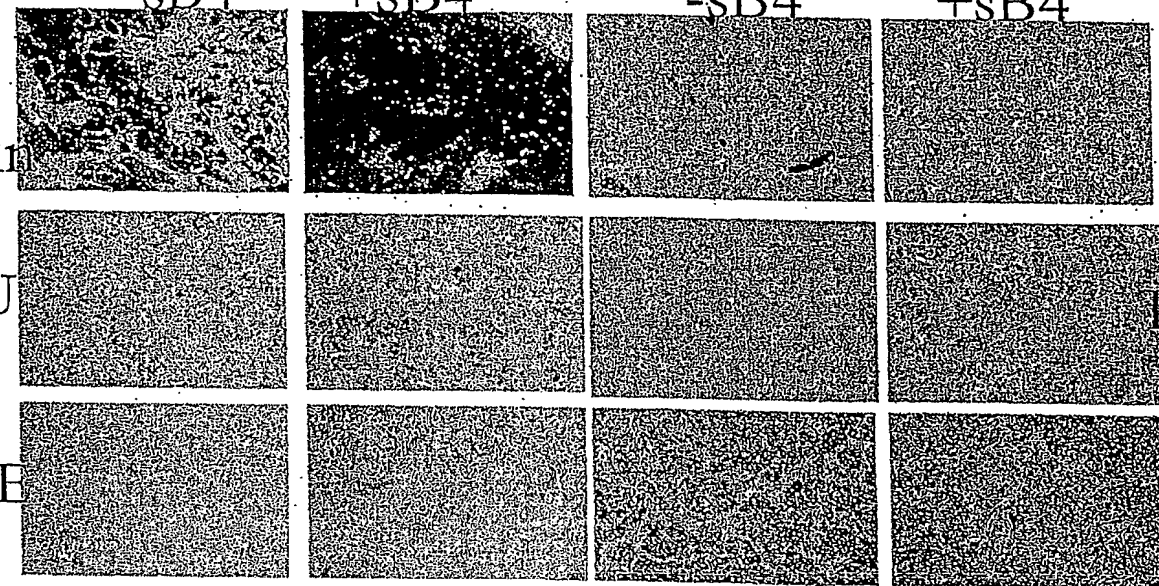
VW

BrdU

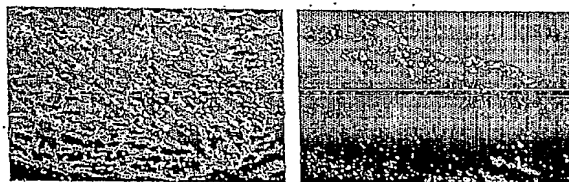
Tunne

H&E

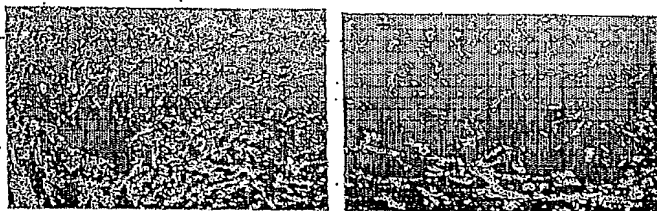
MT



B16



Mcf-7



Soluble EphB4 causes apoptosis, necrosis and decreased angiogenesis in three tumor types, B16 melanoma, SCC15, head and neck carcinoma, and MCF-7 Breast carcinoma. Tumors were injected premixed with Matrigel plus growth factors and soluble EphB4 subcutaneously. After 10 to 14 days, the mice were injected intravenously with fitc-lectin (green) to assess blood vessel perfusion. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with sEphB4 displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 27

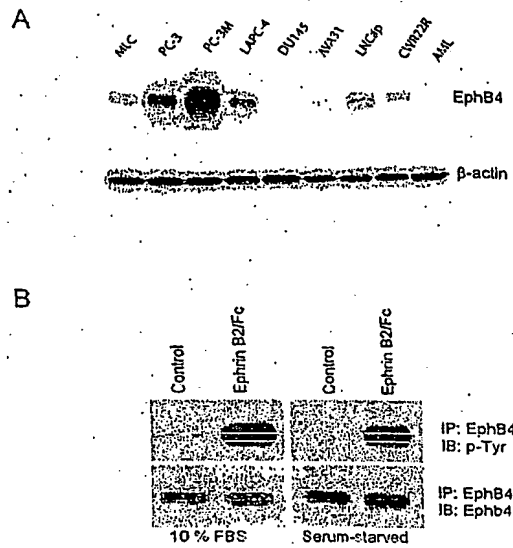
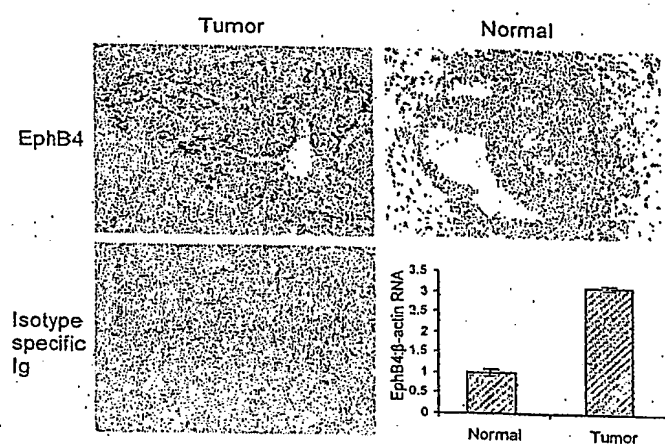


Figure 28



EPHB4 staining in prostate tissues array		
	negative	positive
Normal (n = 20)	17	3
Tumor (n = 32)	8	24

$P = 3.8 \times 10^{-5} \chi^2$ analysis

Figure 29



Figure 30

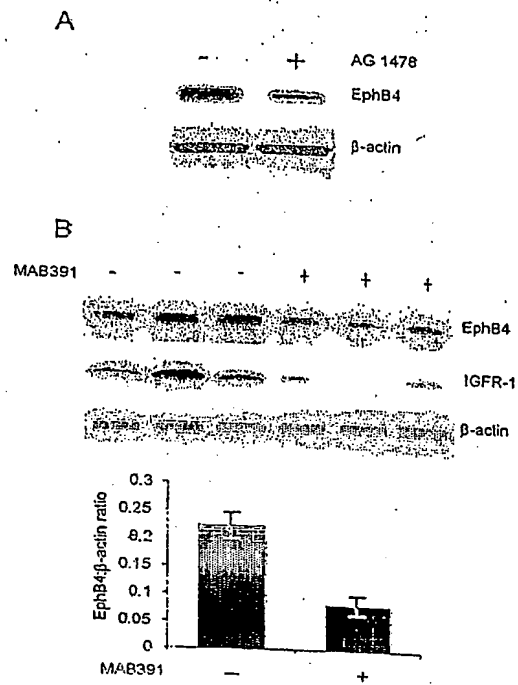


Figure 31

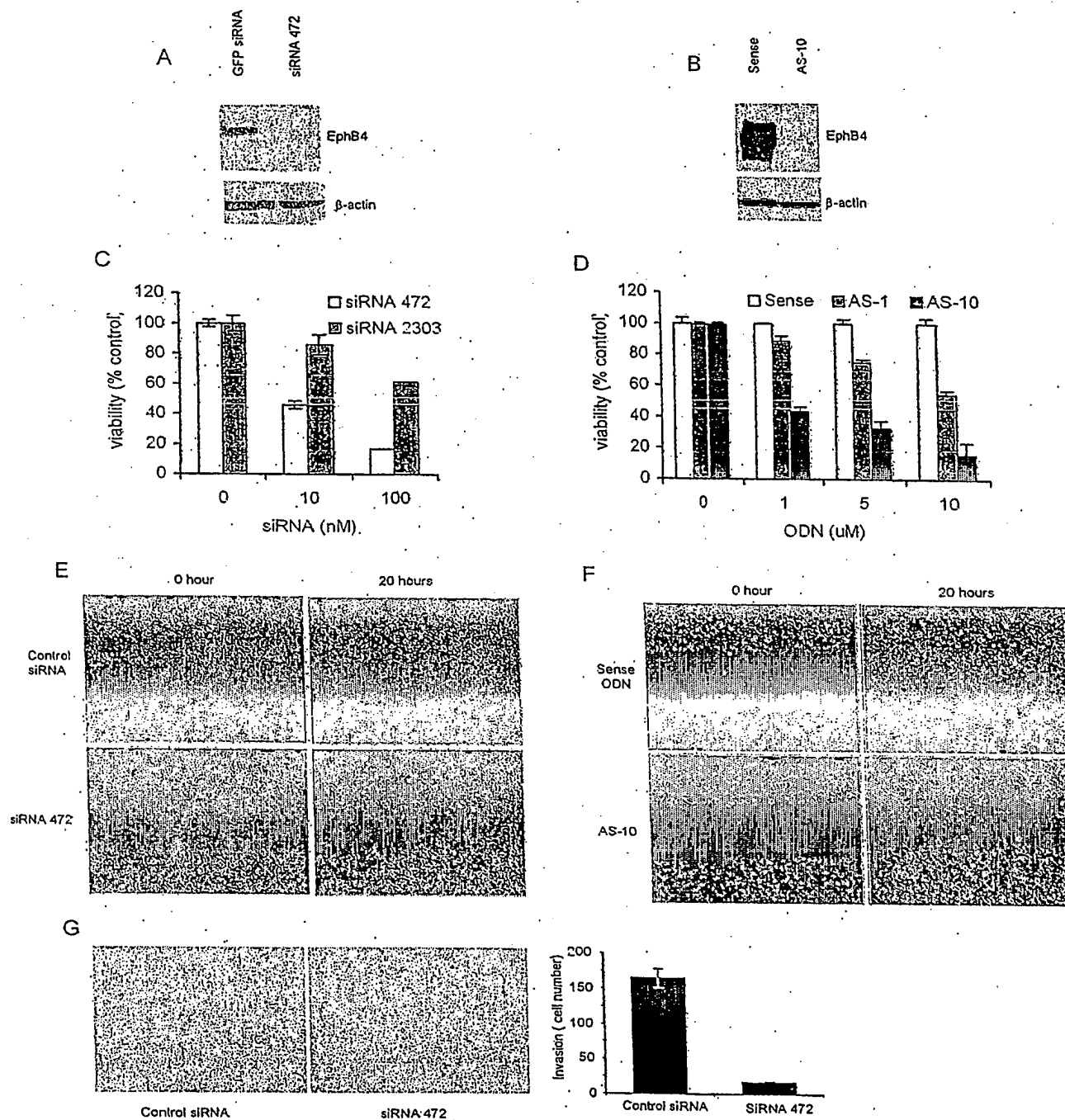
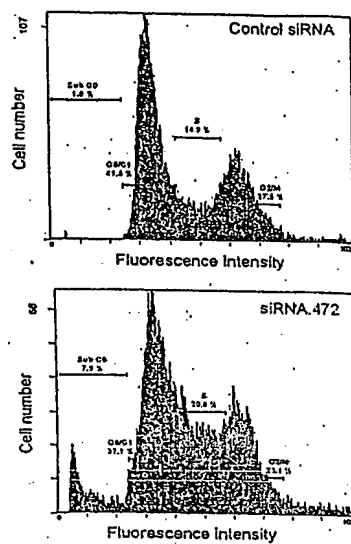
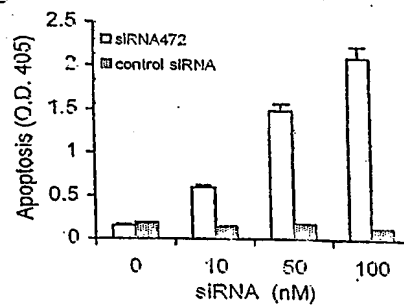


Figure 32

A

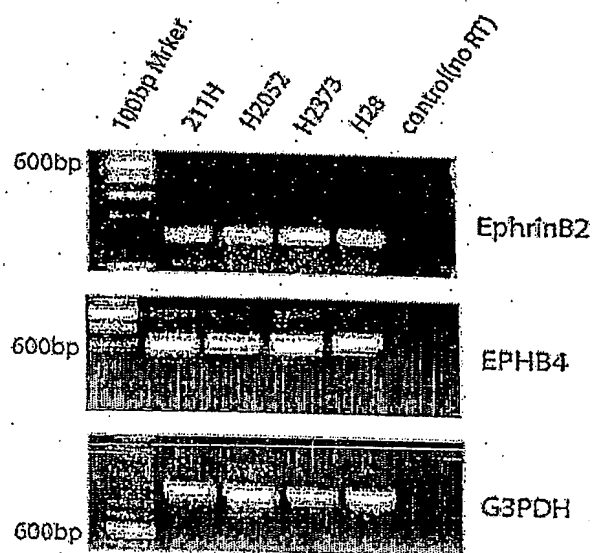


B



Figures and Legends

A.



B.

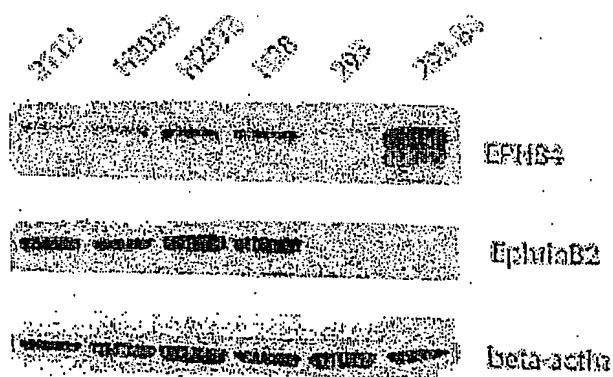


Fig. 33

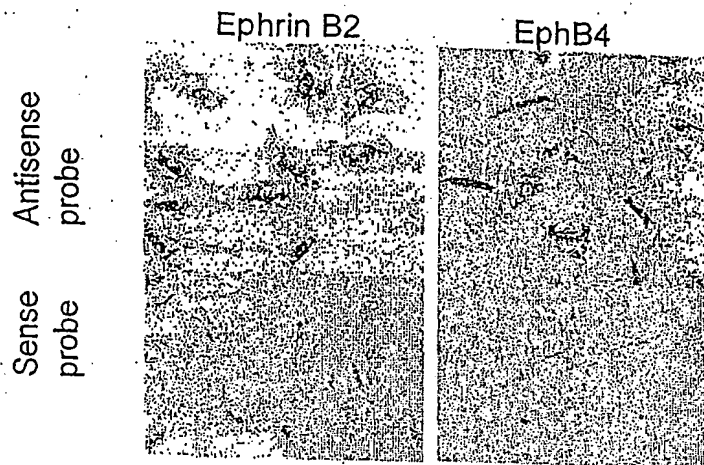


Fig. 34 Expression of ephrin B2 and EphB4 by in situ hybridization in mesothelioma cells. NCI H28 mesothelioma cell lines cultured in chamber slides hybridized with antisense probe to ephrin B2 or EphB4 (top row). Control for each hybridization was sense (bottom row). Positive reaction is dark blue cytoplasmic stain.

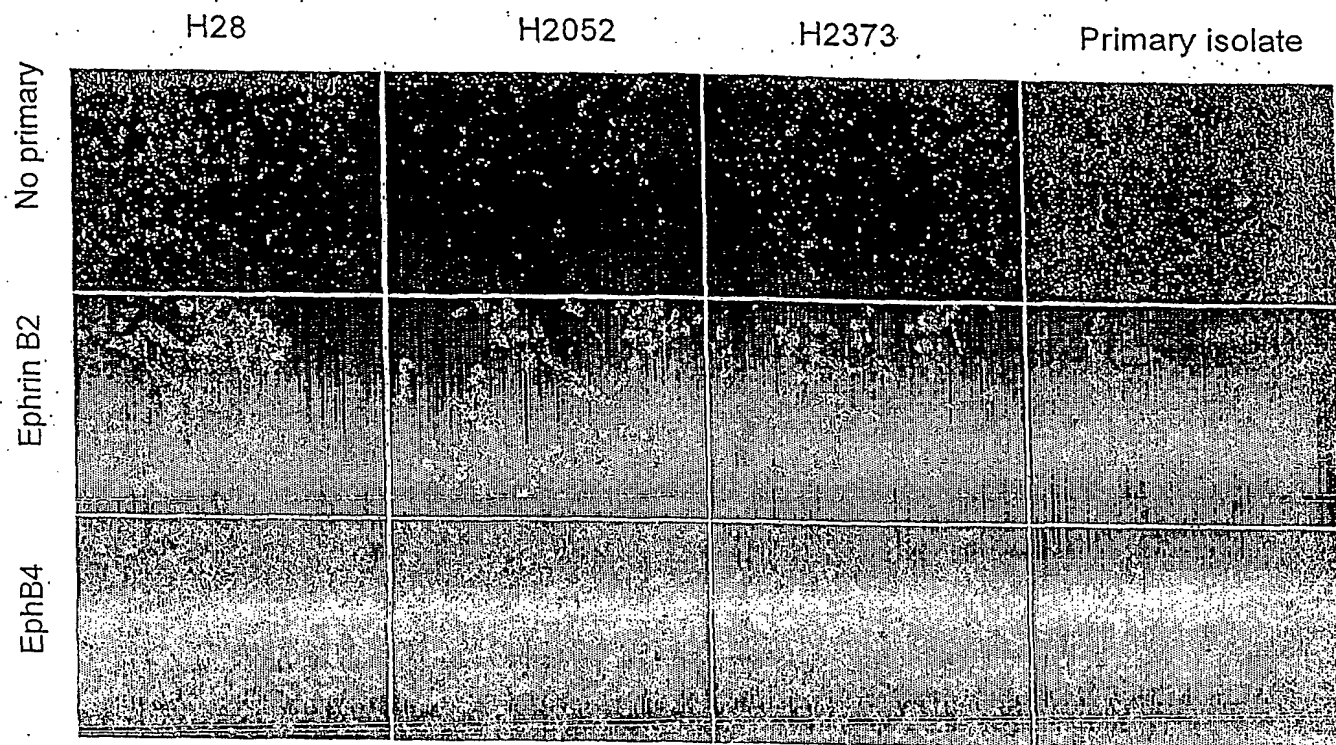


Fig. 35. Cellular expression of EphB4 and ephrin B2 in mesothelioma cultures. Immunofluorescence staining of primary cell isolate derived from pleural effusion of a patient with malignant mesothelioma and cell lines NCI H28, NCI H2052 and NCI H2052 for ephrin B2 and EphB4. Green color is positive signal for FITC labeled secondary antibody. Specificity of immunofluorescence staining was demonstrated by lack of signal with no primary antibody (first row). Cell nuclei were counterstained with DAPI (blue color) to reveal location of all cells. Shown are merged images of DAPI and FITC fluorescence. Original magnification 200X.

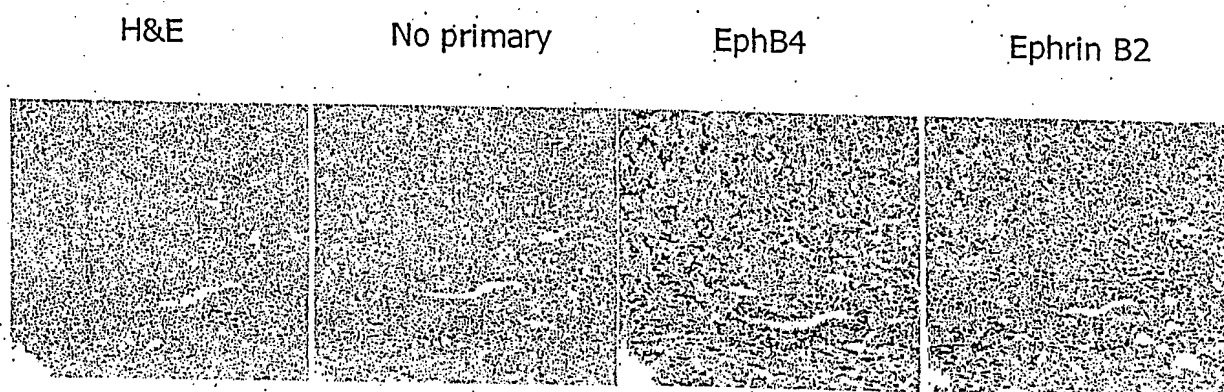
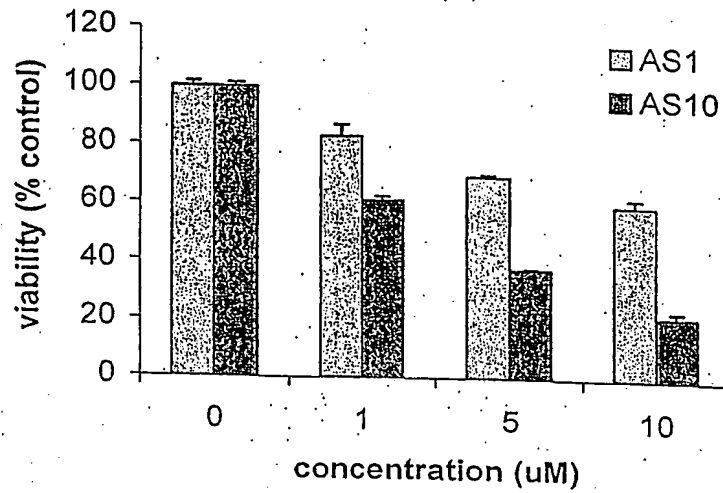


Fig. 36. Expression of ephrin B2 and EphB4 in mesothelioma tumor. Immunohistochemistry of malignant mesothelioma biopsy. H&E stained section to reveals tumor architecture; bottom left panel is background control with no primary antibody. EphB4 and ephrin B2 specific staining is brown color. Original magnification 200X.

A.

Effect of EPHB4 antisense ODN
on the growth of H28 cells



B.

Effect of EPHB4 siRNA 472 on the growth of H28 cells

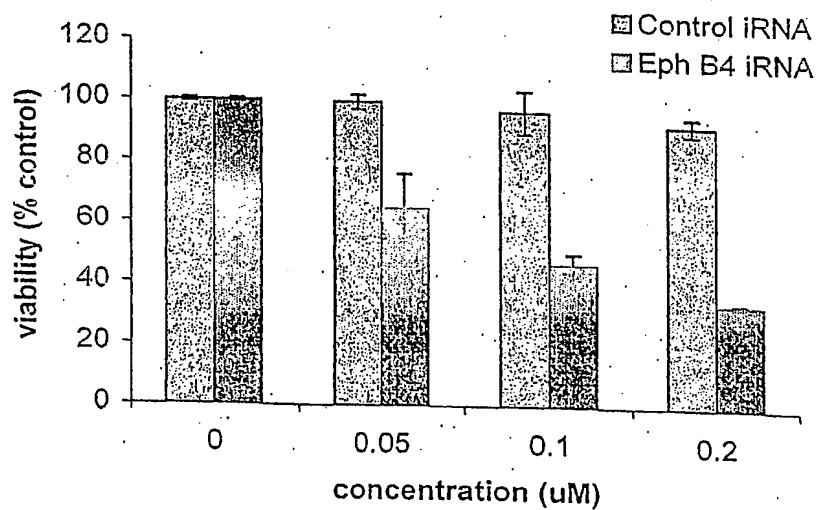
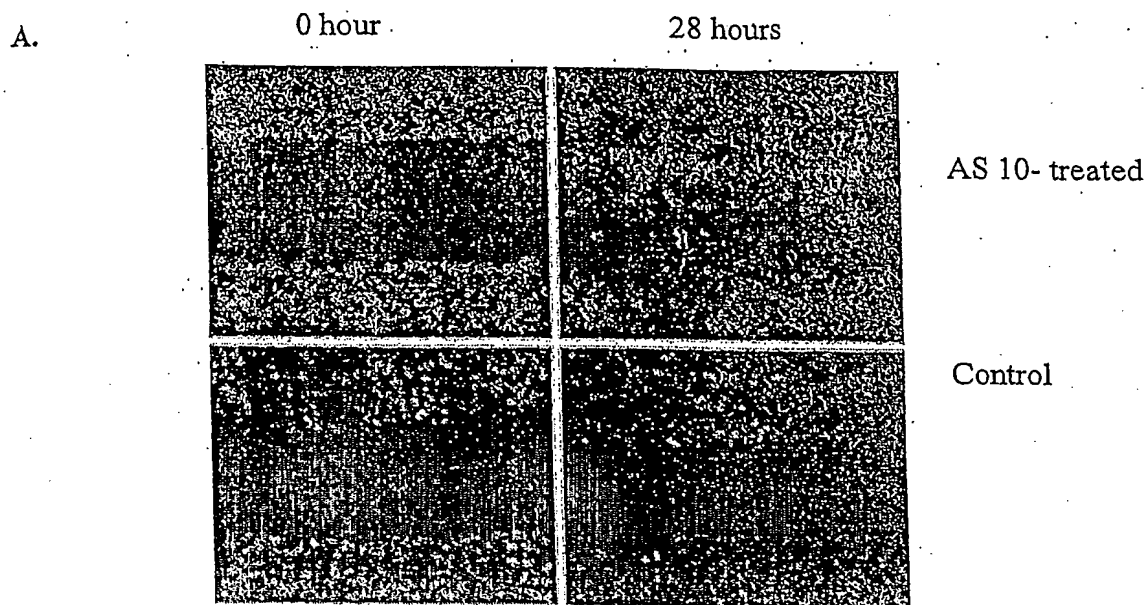


Fig. 37



B. Migration Study of H28 with siRNA472(Boyden Chamber)

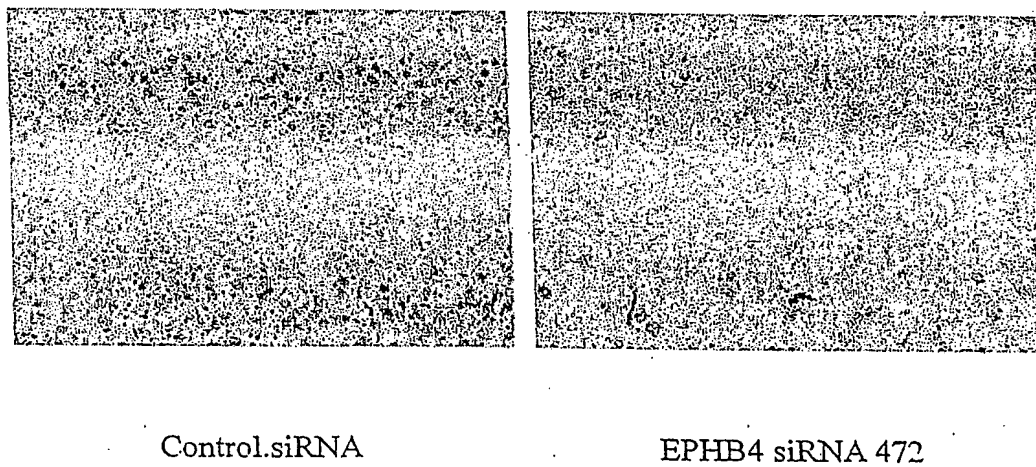


Fig. 38

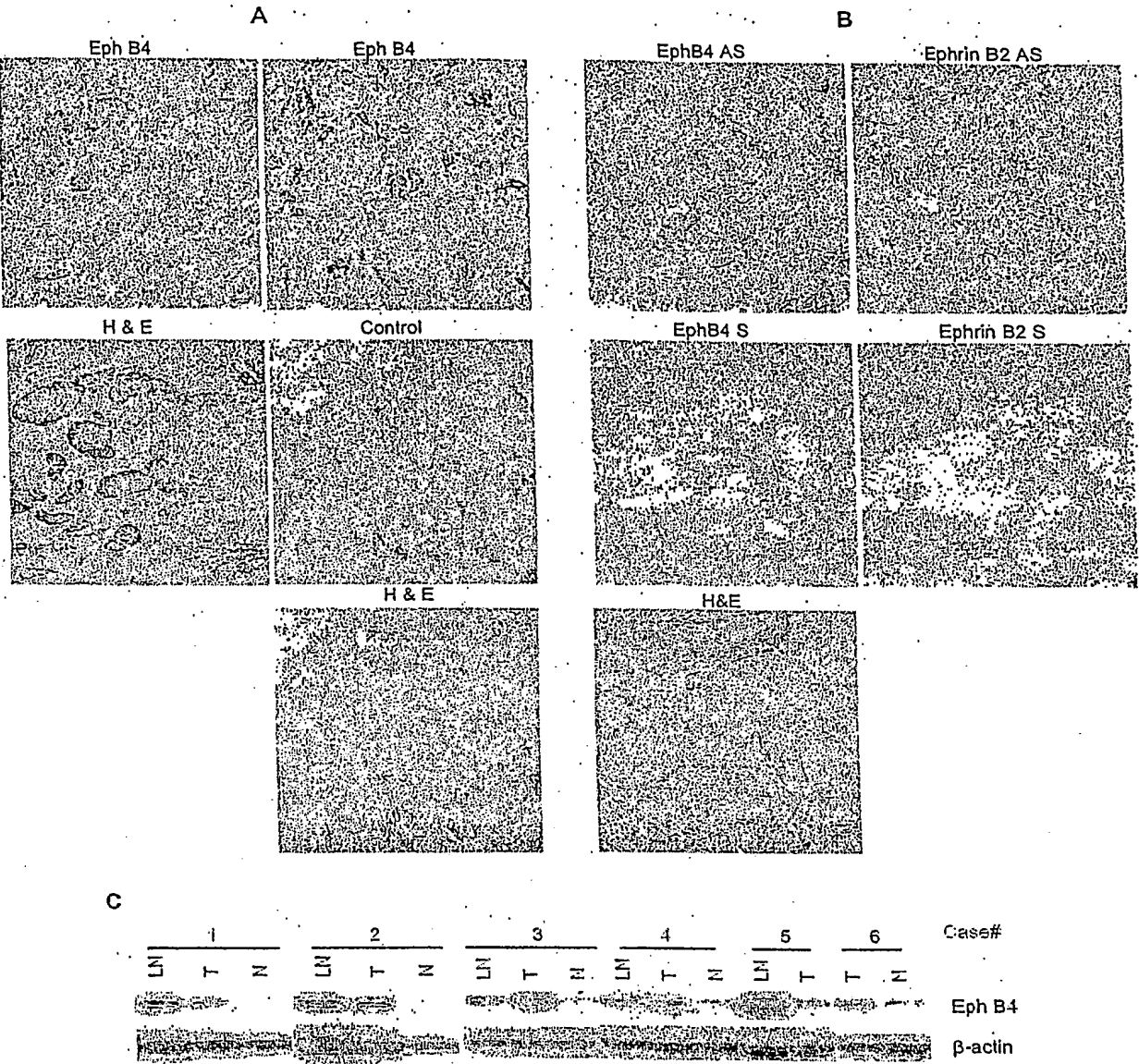


Fig. 39

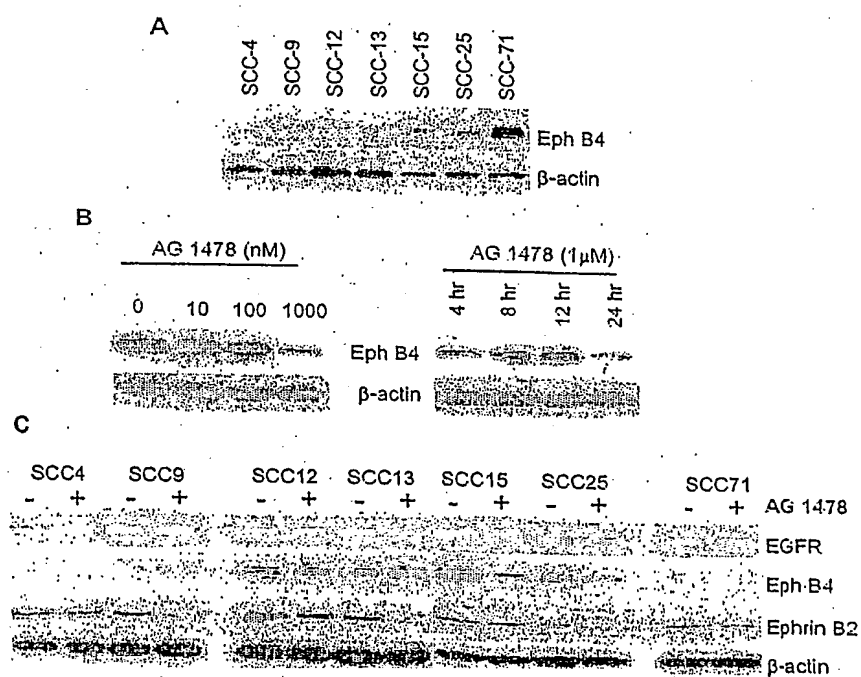


Fig. 40

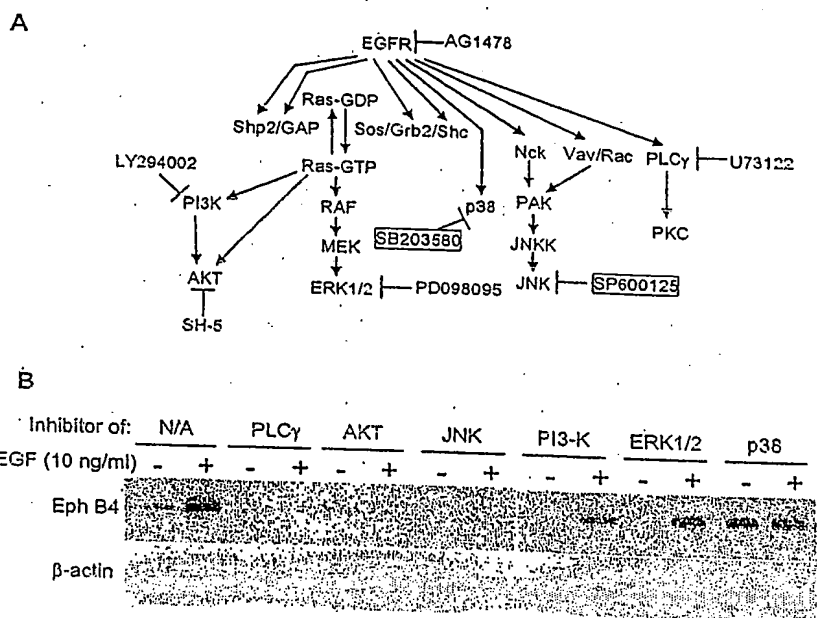


Fig. 41

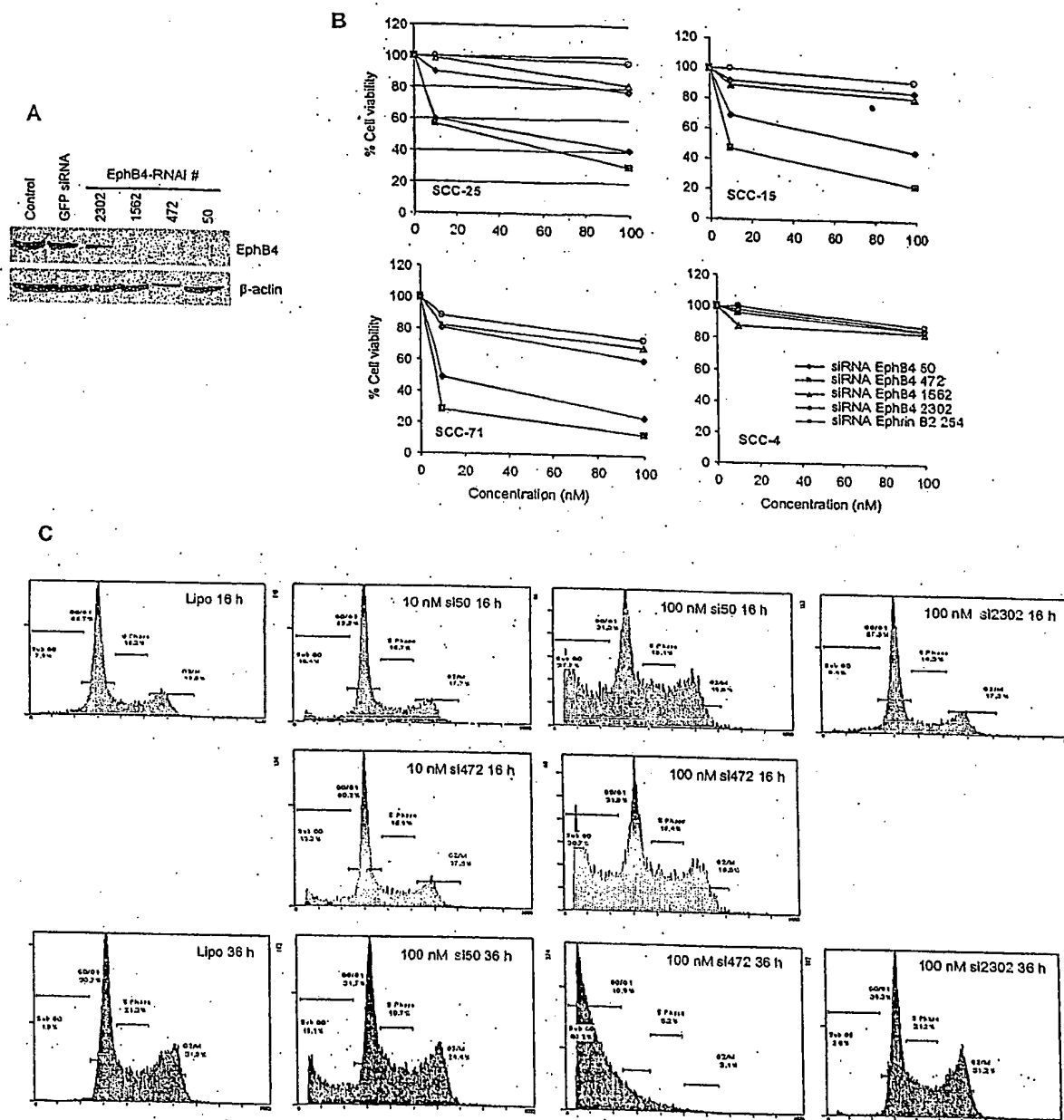


Fig. 42

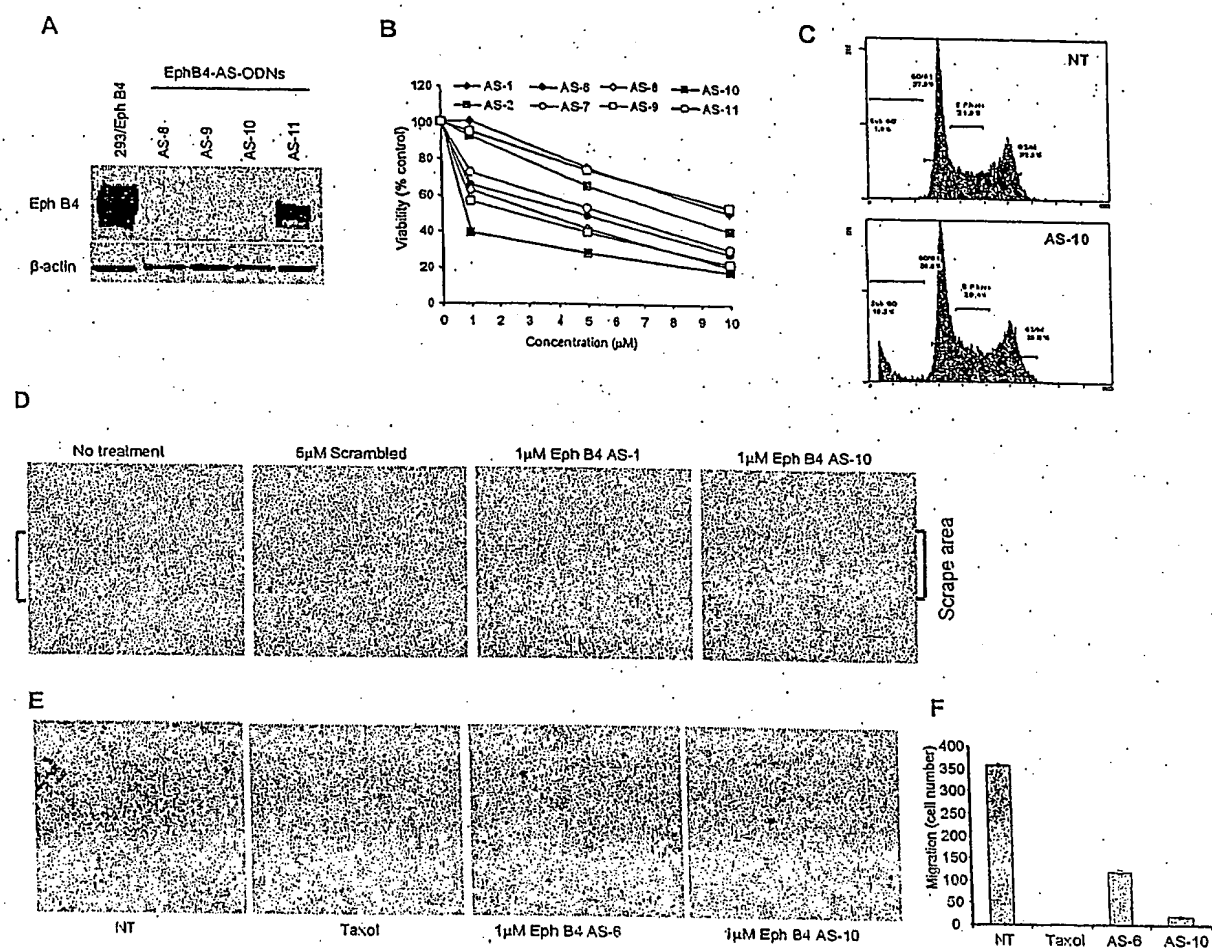


Fig. 43

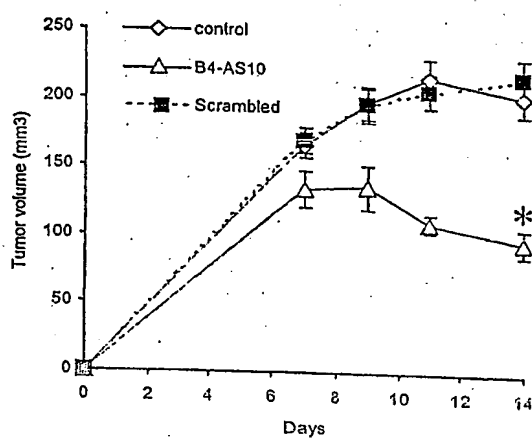
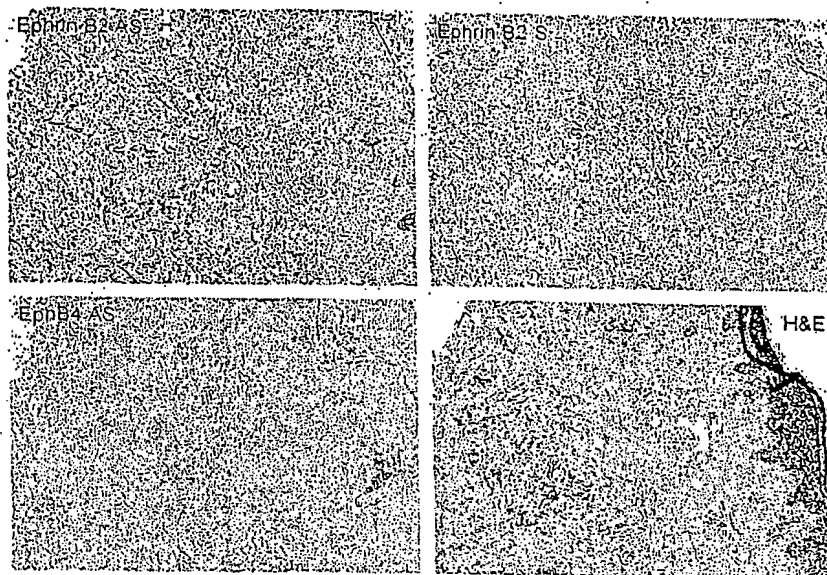


Fig. 44

46/88
Fig. 45

A



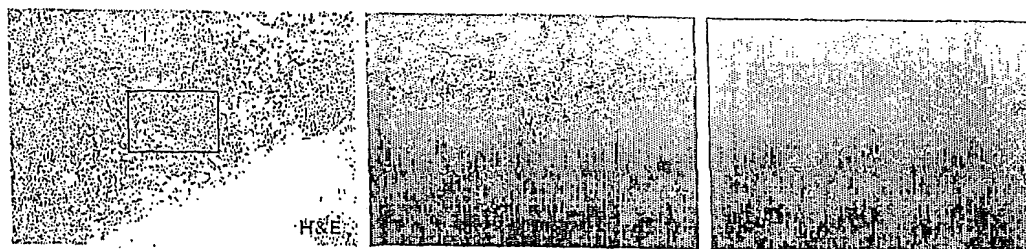
squamous cell carcinoma



EphB4 AS

H&E

B



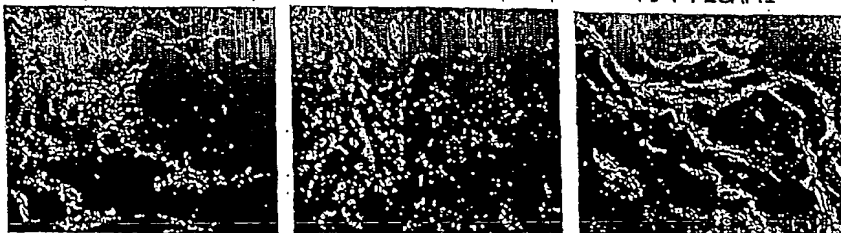
H&E

C

Ephrin B2 + LANA

EphB4 + LANA

PI + PECAM1



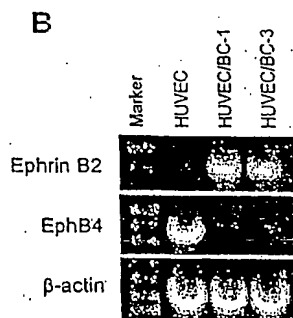


Fig. 47

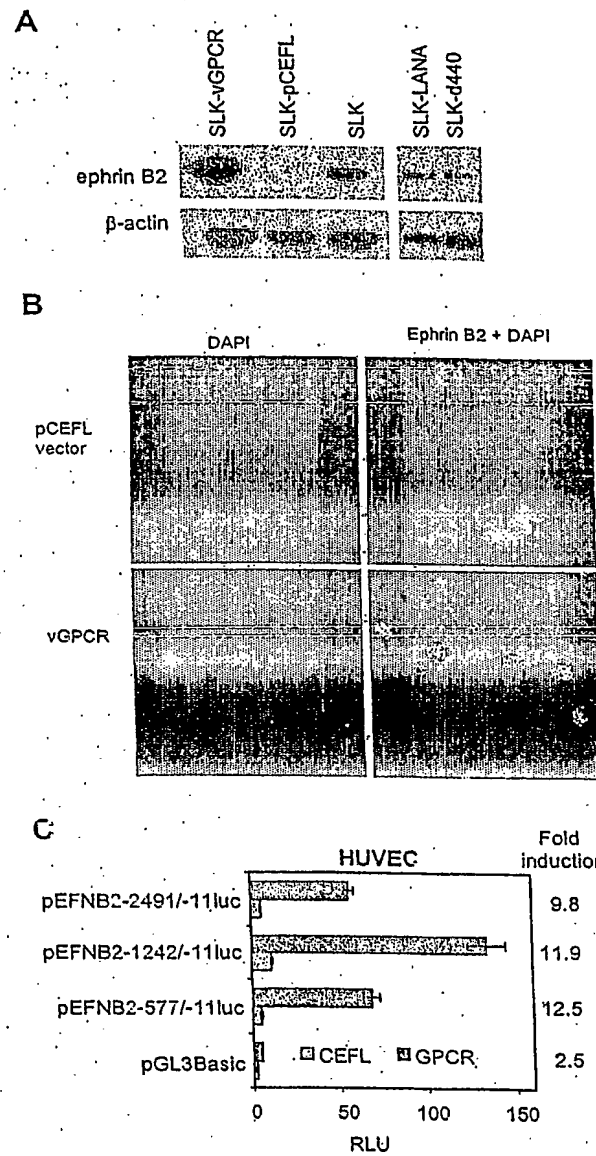
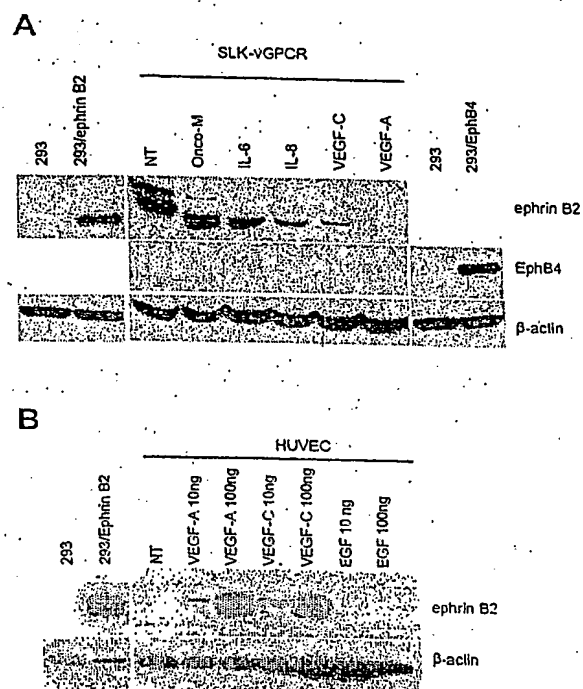
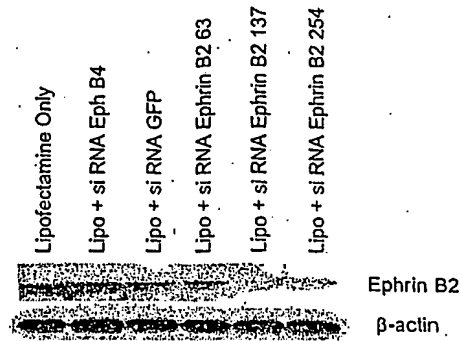


Fig. 48

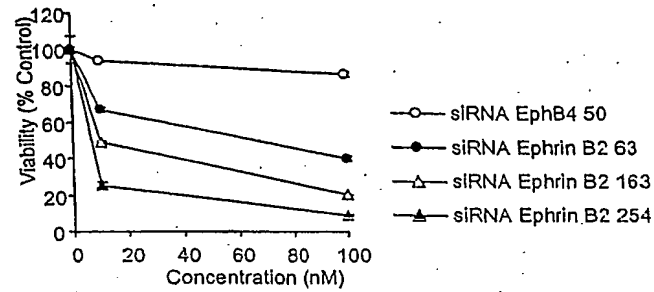


50/88

A



B



C

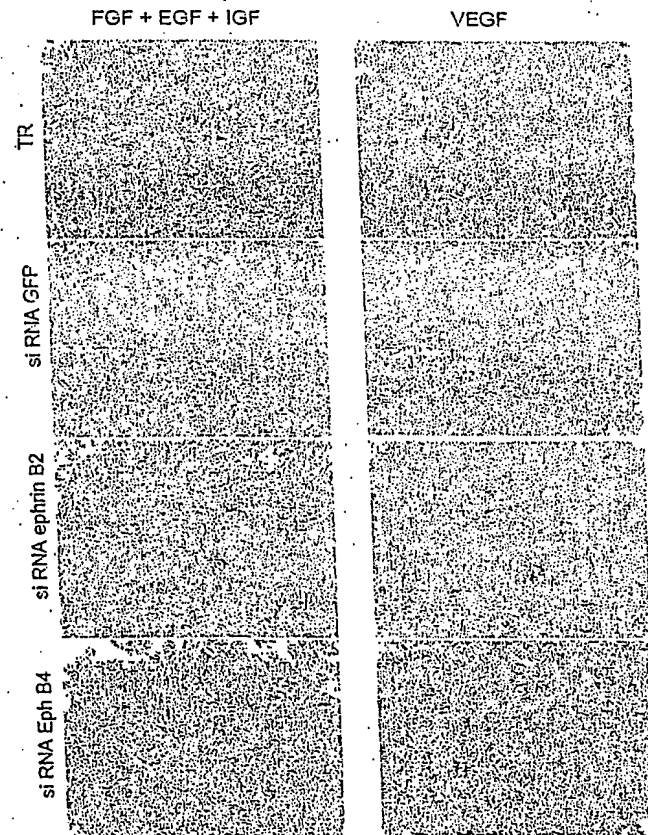


Fig. 50

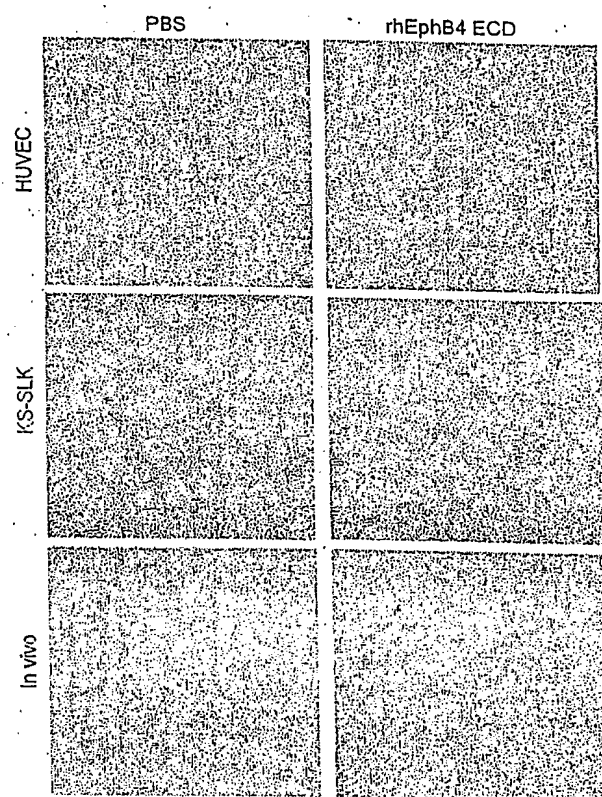
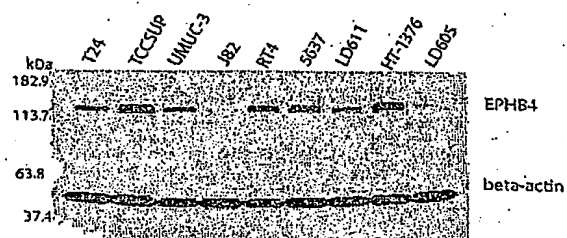


Fig. 51

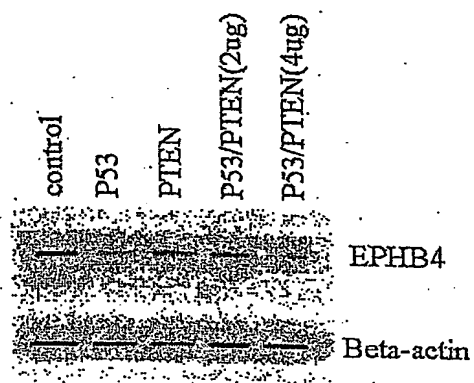
Expression of EPHB4 in bladder cancer cell lines



Regulation of EPHB4 expression by EGFR signaling pathway



Transfection of p53 inhibit the expression of EPHB4 in 5637 cell



Growth inhibition of bladder cancer cell line(5637) upon treatment with EPHB4 siRNA 472



Apoptosis Study of 5637 cells transfected with EPHB4 siRNA 472

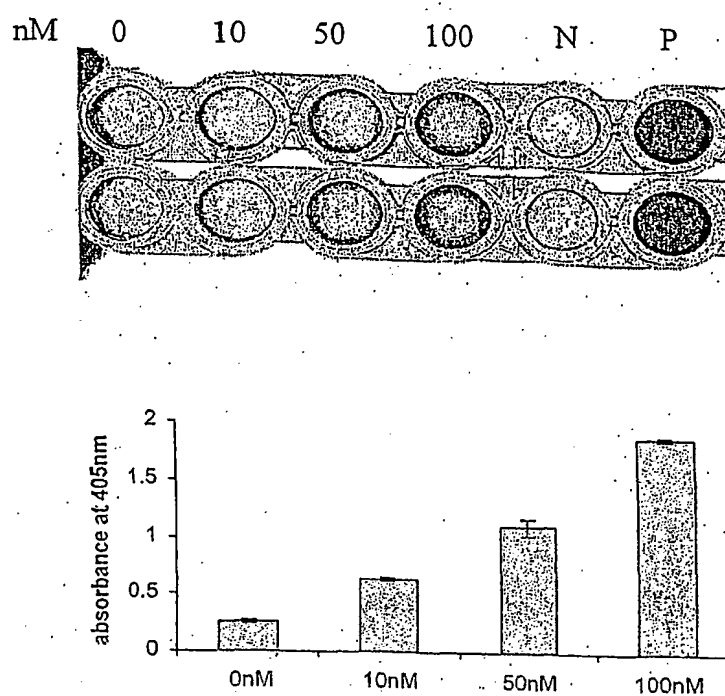


Fig. 54

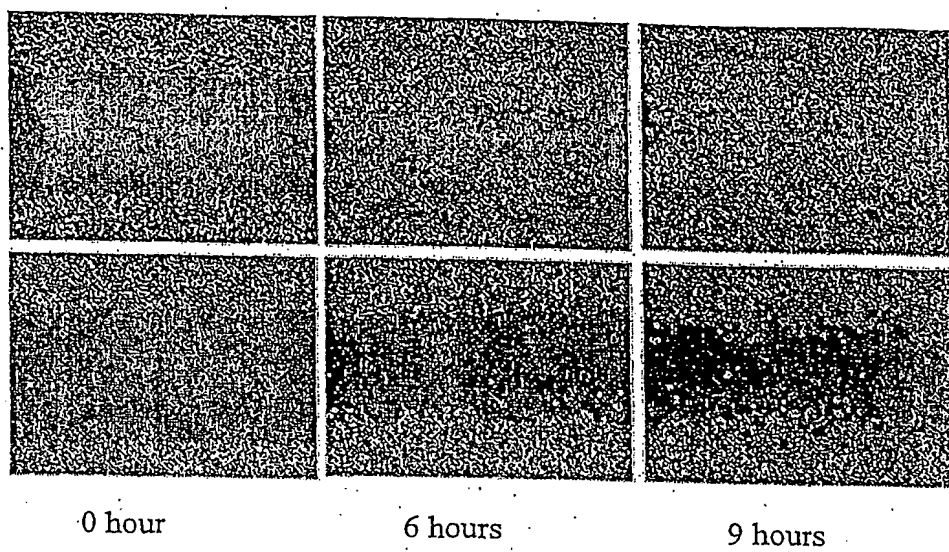
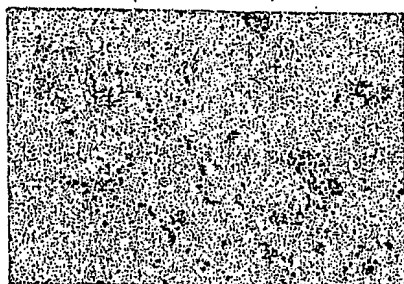
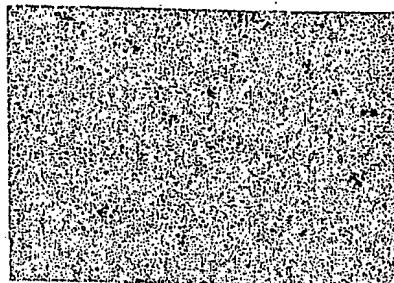


Fig. 55

Invasion study of 5637 cell transfected with siRNA 472 or control siRNA



Control



siRNA472

Co
ntr
ol

si
R
N
A4
72

Fig. 56

Fig. 57

Comparison of mABs by G250 and in Pull Down Assay

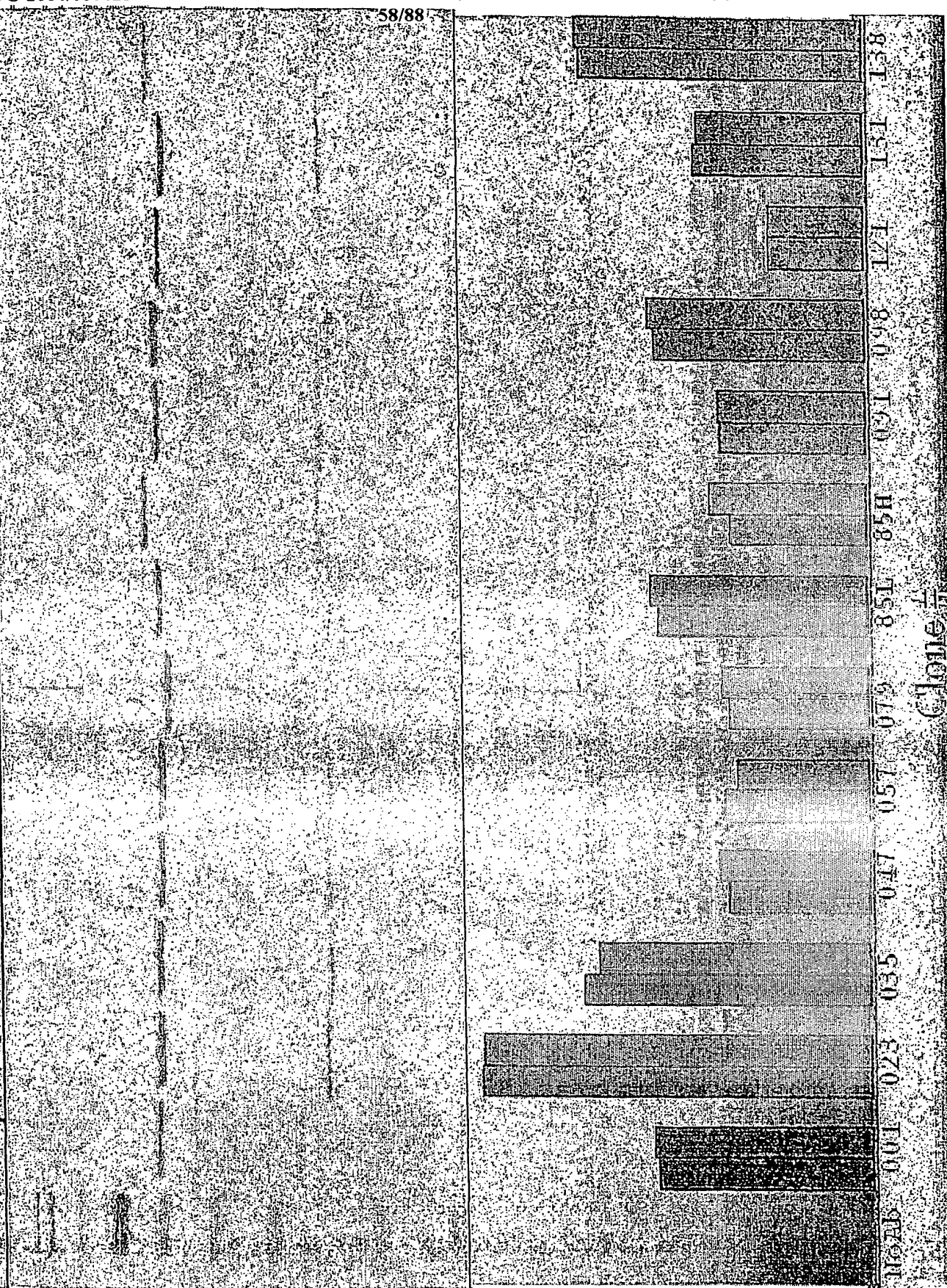
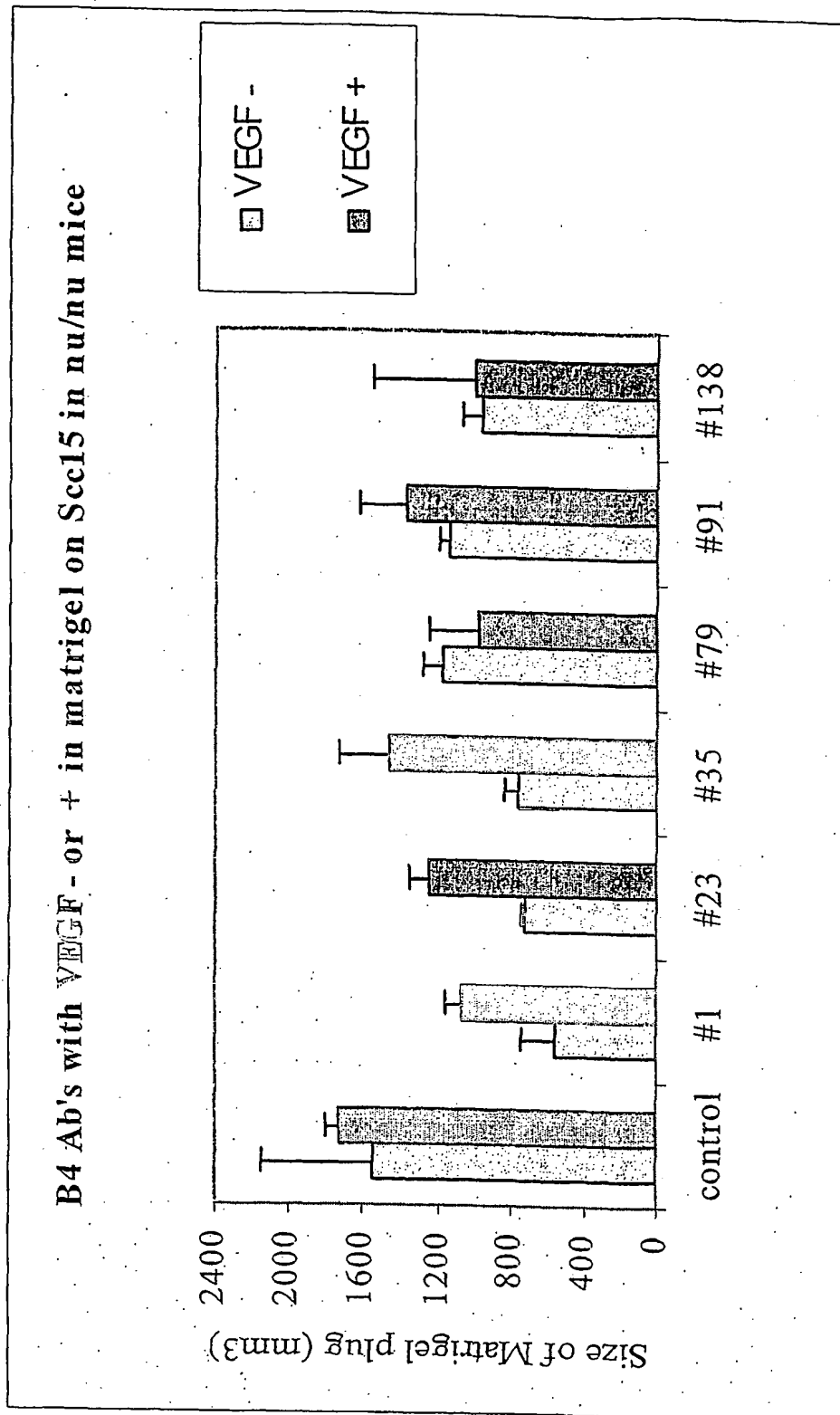


Fig. 58

SCC15/MG xenograft Tumor regression



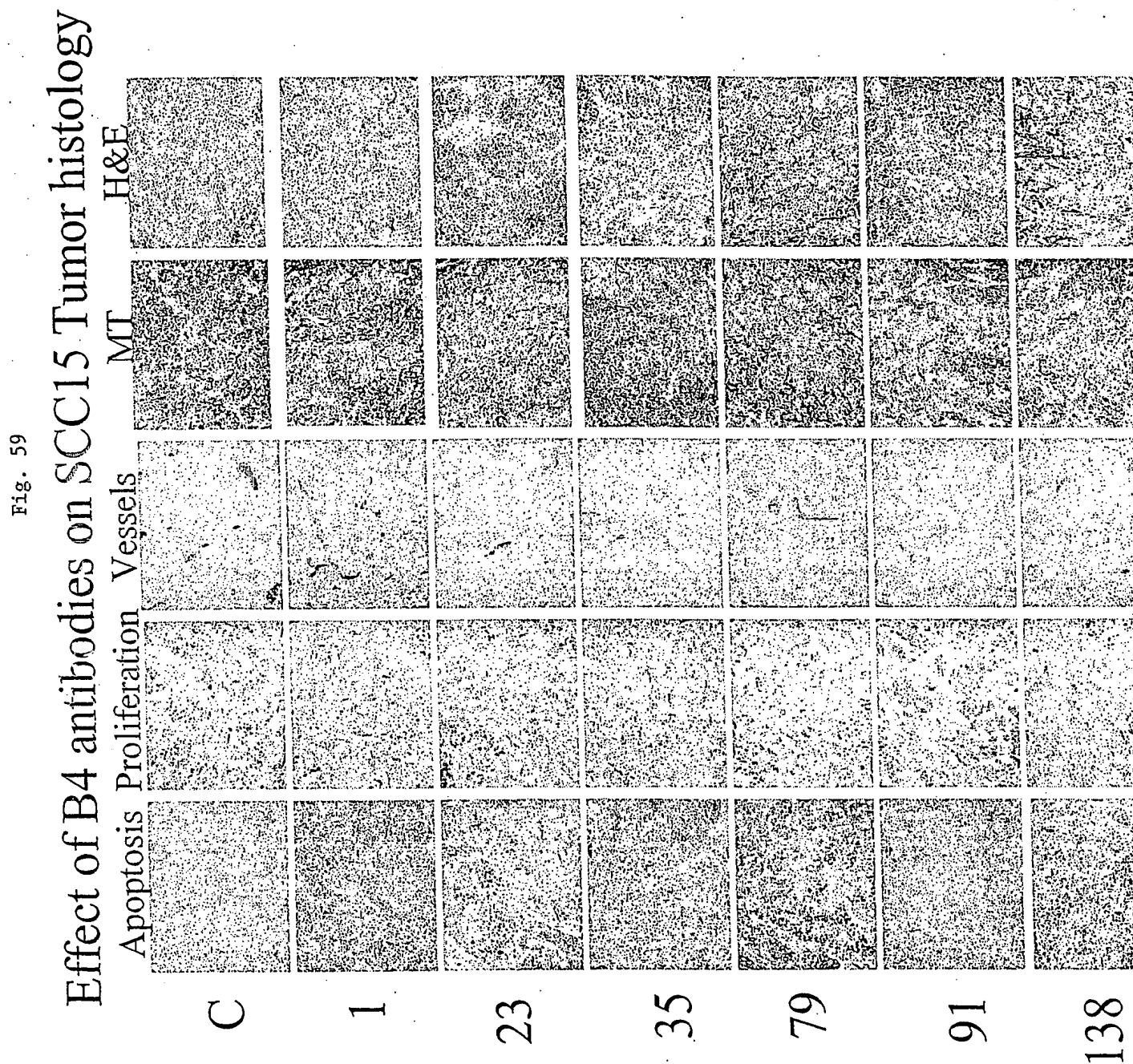


Fig. 60

SCC15/IP,SC B4 Ab treated xenograft Tumor regression

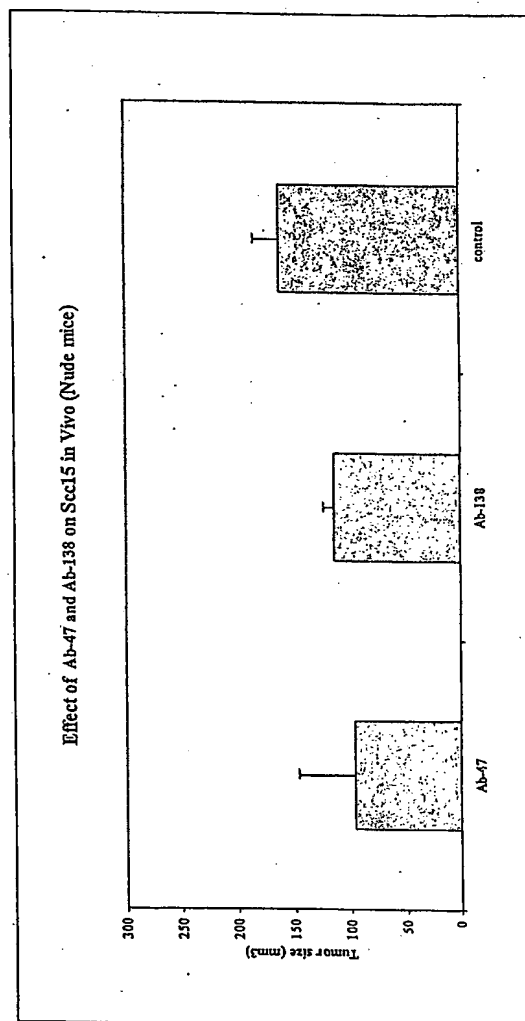
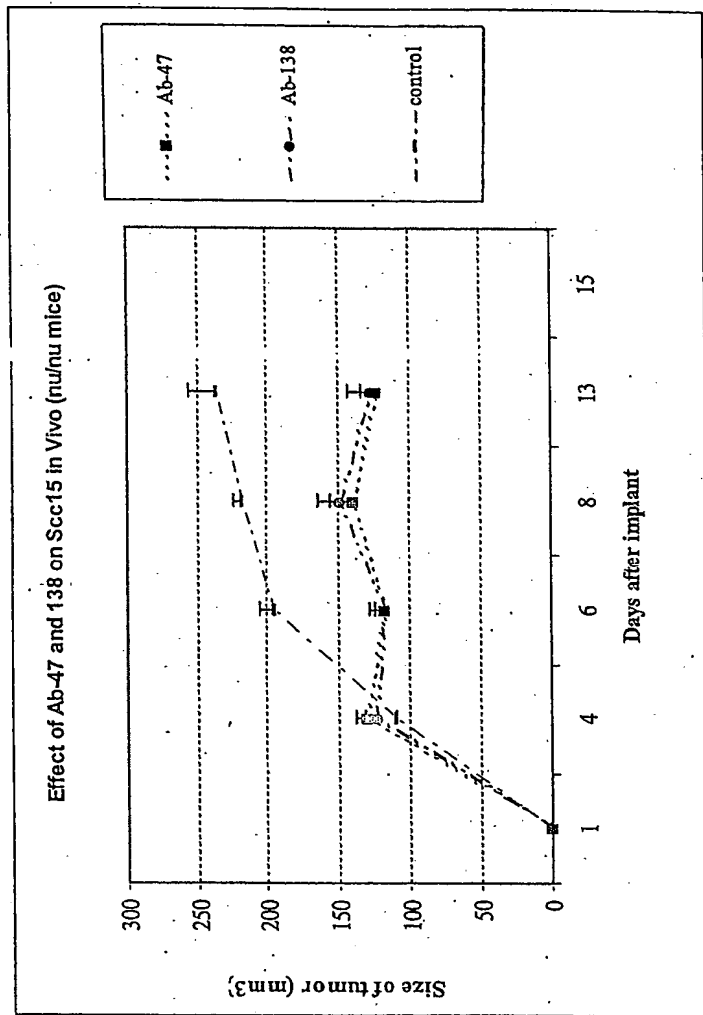


FIGURE 61 EphB4 gene

```

1  ggggtttcat  catgttggcc  aggetggtct  tgaactcctg  acctcaaatg  atccgcctgc
61  ctctgcctcc  caaaatgctg  ggactacagg  cgtgagccac  cgcgcccgcc  acacccacct
121 tttctttacc  gttgtttcct  cgatttttct  ctactcccta  gcgcagctta  gtgcgcgcct
181 cctctggaca  tttttcaggg  ettggttgcg  cgcacagtag  gtccccaaca  ctgaatgttt
241 atggggtgac  tgtgtgaacg  ttcgctgcaa  ggctatccaa  actgggattg  ctcccttgagg
301 cccctgggc  ggccgtcaat  tctccaaagc  ttctactccc  ttttccttcc  ttttcccca
361 aaacgcagtc  cctgcgcccc  ctagaggggtg  gtgggcgcac  ccaagagcgg  catctagagt
421 ccgcagcaag  gtcagagcgg  gctttgtgtg  cgcggtgaac  atttacgtgc  acgcctgggc
481 ggccctccgt  gttgctgctg  ggtgtgtgtt  ttctctgctc  cctggtgcca  gccgggttcg
541 ggctgtccc  gggggtccct  gggcccccagc  cccgacatgc  tcggtcctgg  acagcgcgca
601 ccgccacggc  gcacatctgg  gcggtcccg  gggtccctcac  ccgcgcgcc  tcccccttct
661 ccaaactttc  tctcaacttc  ccgacctgct  ccactcgggtg  ccctctccg  ctccctcat
721 gaattattca  gtacgctgag  ctccaatcag  cgcgcgccgg  gctcactcgc  ggagcccccg
781 cgttgggaga  gctgcccccg  ccccccgcgc  gcccctccct  cccgggccc  gcgcgcgcc
841 gcccagttcc  agcgcagctc  agccccctgc  cggcccgccc  cgcgcggctc  cgcgcggctc
901 tctccctccc  tcccgctccg  tccccgctcg  ggctcccacc  atccccgcc  gcgaggagag
961 cactcggccc  ggccggcgca  gcagagccac  tccaggagg  gggggagacc  gcgagcggcc
1021 ggctcagccc  ccgccacccc  gggcgggacc  ccgaggcccc  ggagggaccc  caactccagc
1081 cagctcttgc  tgcgcgccc  cccggcgcg  ccactgcag  cagctcccg  gccgcgcgc
1141 cgcgcgcgcg  gcacagacgc  ggggccacac  ttggcgccgc  cgcgcgggtg  cccgcacgct
1201 cgcagtgagg  cgcgctgagg  gcccgcagca  ggagtcctgc  gcggagtatc  gggtccacc
1261 cgcagtgagg  gaggcagacc  tgggggggg  agggccccc  aaactcagtt  cggatccac
1321 ccgagtgagg  cggcgccatg  gagctccgg  tgctgctctg  ctgggcttcg  ttggcccgag
1381 ctttggaagg  tgagtttct  tgccgggggg  ggccacccc  gtactcctg  ggacctccc
1441 cccaacatct  gggcctcgga  gtggaggggc  cggcctctga  ctacctctac  ccgggactg
1501 cagtccaaa  cacttcggac  cgatagtgt  ggaacgggag  gggggcggg  aagaggcgcc
1561 cgacgggtag  tggagttttc  tttgttttg  gaaagagatg  gaggctgggt  acgaccggg
1621 acattccct  gcccgggctc  cccgaactct  cactgctgat  tacatacgcc  cctggctgcc
1681 tttcctttcc  tccctacccc  actattcaaa  actatctgca  aagtttctgt  cctgctcca
1741 cctcccgccg  tacatgagg  aaggtttctg  gagaagcaac  agcagacaag  gcacaacttt
1801 tctgtctagg  ccctaaaacg  acccccagcg  ccaattcctt  agcgatcaca  ccttgatcct
1861 ccagttccac  actcctgcaa  caggatggcc  tcctttgcat  tcacacagca  aacccccaaa
1921 ccgctctccc  gccactgct  cctgcccctg  gtatagggtg  gctccttgg  ttctacaggg
1981 tgcaccccat  ccctttaaat  gcggtctaga  ccccgcccc  aggtgagtc  cgggttccc
2041 ttgagacct  ggagcgggta  gaaactgacc  tacacagccc  ccaggtagaa  actgacctac
2101 acagccccc  catcgcccta  actaaccag  tctatctccc  acctcctggt  ctctccaacg
2161 atttctttgg  ccattgattg  ctgtccctcc  tgggtcccta  aagggggagc  caagagccct
2221 agaaactctc  ctgtgtccct  aatgtccttt  cagtgagctg  ccaacacccc  cctttctctg
2281 tctgggtatg  aagtgggtat  gggcgggtag  gctatgagg  actcccaaag  ggaaggattc
2341 agcggcggt  gaaaaaccct  ctccccctgg  ctgggcagga  ctgcccctgg  ctggggatca
2401 aaggctaggt  gtgggggttg  gaggtagggg  aggcttgccc  agctcagaga  acggagaagg
2461 gggaaacaaa  accatgaacg  aggggaagag  gaaggccaaa  ggggtggaaa  aaccacgagg
2521 acgaggtgtg  gtgagaagga  aagacgcaaa  gaggaatgg  tgattgtgac  acctattacc
2581 tgagtgtttc  caagcaccag  gctgtgctg  aggccttac  aaatattaat  ttcacccatc
2641 cagcaacgct  aagggtgggt  ctattattgc  cccattttt  cagatgagga  ggctggggct
2701 tagttaaggt  taagtgttt  atccaaggcc  ctgtgccgcg  aggaacagcg  agaagtggag
2761 gccgaaagcg  aaggagagat  agtgactgtc  agaaagagaa  acggaggtgg  acagagagtg
2821 gaggagagat  aggtgagaga  catgcgaact  gacagatcaa  agcgtggctg  cagctgagct
2881 gggacgcaga  aaggagagct  gcgttgcctc  tgggctgcgg  acagcccgag  gcagagacag
2941 tgtgtaaatt  ggagacagga  aaacactatc  ccggctggaa  caatggagg  tggagacggc
3001 agcctctatc  caccctcttc  ccagacccc  ggcacccgtg  cccagtgag  cagggctgtc
3061 tcttgccacc  catggggacc  ttgcgcctct  cacctcaggc  tggctggctt  cccatctgac
3121 ccctagctgg  aggacatcat  ttggtcccca  ggaagaggct  gcctcaccca  ccctctttct
3181 cttctctct  gcagctccca  tgggggtggg  gccaggtgtt  ctggctcccc  tctccacct
3241 tccagcgcc  caatgcccc  cacattgccg  gcccccaggg  ggattcctgt  accctccctc

```


3301 ctccactctc cactgccagg ggctgtgcag tttttcctaa tccccccct tccccaagt
3361 cctgtccctt ccccgatga tccgagccaa gccagggtgtg ttcacccctc ccattcatac
3421 cgccccccag aatctcctcc cctctgcctt cccataacca aatccagatg tgaggcctcg
3481 gcgggagcct gggaacccta gcatcccgac ctccagtgtt tcttgatcag ggcactcgtg
3541 gggaggaggg tactgggatg gggggccagg ctatgcccc ggcacggagc gctcccttca
3601 aggagggaag gacggggtgt ttgggtctgaa agcagagagg ggtcttggac aggggaatgaa
3661 attgtggggg agagaggctg attctgggac ttagggggagg aaacgtggag gctgagacaa
3721 gaggttcccc tcccacacca gcagcctctg ctctgggggg tcaggaccag ggcgcagctc
3781 tcattttaac cctttctgag ctgcccggcc ttctcccgtt acattttgat ctccctccct
3841 cctccaggga ggcctagatc tggggatcc caaggagacc ccatgcctac cagatgttgg
3901 ggggtgggggt ggcacttagc agaagaggcc agaaatcagg cgggtgcaga gggcagggtc
3961 tgctccctc ttggcccccc aactcctcta gctcagagct aagaggatcc acctgcctcg
4021 gttcccaggg atctggtctt cctgacctcc ctccccacc ccaggcactg actctgtctc
4081 tctgtctgtc tcagagacct tgctgaacac aaaattggaa actgctgatc tgaagtgggt
4141 gacattccct caggtggacg ggcagggtgag agctgcaccc aggagctgga gctctggagg
4201 gaaactgagg gaggagagg cgctgtgccc gctgtcttct tgtgtgccac tctctccc
4261 tgtccccca gatgacagca gcccagcag tgtcgtctga gcccttctca gaggcgcct
4321 cctcgcagta ccagcagccc ccttttctca gtccctctca ctttatagga ttcacccat
4381 gcagccctct ccctggcggc tcccagccc ccttgtgtac ctcttctct gcacagtggg
4441 aggaactgag cggcctggat gaggaacagc acagcgtgcg cacctacgaa gtgtgtgacg
4501 tgcagcgtgc cccgggcccag gccactggc ttgcacagg ttgggtccca cggcggggcg
4561 ccgtccacgt gtacgccacg ctgcgttca ccatgtctga gtgctgtcc ctgectcggg
4621 ctgggcgctc ctgcaaggag accttcacc tcttctacta tgagagcgat ggcgacagg
4681 ccacggccct cacgccagcc tggatggaga accctacat cccgtggcct ggagggcagc
4741 agggctcagc cacagccaag gtgggattcc agccagcagg cccgtggcct ggaagcagga
4801 cgatgtagtt gcgaggcctc tggccgcgc gctgggggt ggaagcagga ggcttaggtc
4861 tggggaggga agggggtgat cttctgggcg gaggagcaga atatacgggg gctgcctggc
4921 cgggccccca gggaggccca agggtcaggc ttctcctcca gtccactcaa cccactacc
4981 ccaactgtgt ccagccacac tgagtttctc ccattccctg actgcacctg gctgggttcc
5041 agctcaagac ttgacagcg tttgcagcg gctcccaga tcttgctgt gccaccttg ctgactctct
5101 tacttgtctt cggagtcca gctcccaga cctgtgtcca cctgctgtc tgggactca ttttacttta
5161 cctccctaca atcctgcata cctctgtcca agcggggtct tctacgttac ccaggctggg
5221 tttatttttc ttttatatct atatttttaa agcggggtct tctacgttac ccaggctggg
5281 ctctaactcc tgggctcaag agatttctcc cactctcgcc tccctaaagt ctgggattat
5341 aggcagtagg cactacgccc ggcctcatgg tactttataa cttccccagg attcattcat
5401 cgctgtctcc ttgactctga ggtcaaggcc tggcatggcg tcagtgtcag taaagtgttg
5461 tagaacagat gaataaaaag ggggagagg ctagggccaga ggccgggcat atcgcaggag
5521 ctttgcaagg ctgaatggac agtggtgggg cctgcagaaa gtgtgcccgt ggggaaggtg
5581 agggaagatt ctggaacggg aaccaaggag gtccgggagg gtgagctggg aagaacacaa
5641 cagtccgctg ggtcctcagg gagtggggac agcagcgggt tgccctcccc ccgcggcgag
5701 gtggacacgg tggccgcgga gcatctcacc cgggaagcgc ctggggccga ggccaccggg
5761 aaggtgaatg tcaagacgct gcgtctggga ccgctcagca aggttggtt ctacctggcc
5821 ttcaggacc aggggtgctg catggccctg ctatccctgc acctcttcta caaaaagtgc
5881 gcccagctga ctgtgaacct gactcgattc ccggagactg tgccctggga gctgggtgtg
5941 cccgtggccg gtagctgcgt ggtggatgcc gtcccgcgcc ctggccccag cccagocctc
6001 tactgccgtg aggatggcca gtgggcccga cagccggtca cgggctgcag ctgtgctccg
6061 ggggttcagg cagctgaggg gaacaccaag tgccgagggt agagctggag cttccctgc
6121 gactgctgct catccggggg agagtctga actccactca ggaccactt ctttaagttt
6181 cattttgtat agttagatgt tgaaatggag gcttgcctct gcctcccggg tccctgttca agcagttctc
6241 tggcacaatc tctgctcaac tgcaacctt gctgggata caggcacag ccaccagcc cggtaattt
6301 ctgectcage ctctgagta gctgggata tggttcgcca tgttggccag gctgggtctg aactctgac
6361 ttgtatttta gttagagcg cggcctccca aagtgtggg attacaggcg tgcgtacca
6421 ctgaagtgat ttgcccgcct agactttatt ttcacctgaa attcattaat ttcacttga
6481 caccagctg gaaaaaaaag agactttatt cacttgggcc ccatggaaat cacaggattt
6541 aattccacct gcagttgtag caggacctga cacttgggcc agatgccaag gtgggaggat
6601 gcctgacaca gtgggtcatg cccatagtg ccagcatttg ggtgacaga gcaagacccc gtctctaaaa
6661 cacttgagcc caggagttcg agatcagcct ggggtgacaga gcaagacccc gtctctaaaa

```

6721 aaaattttttt tttttttttt aagacagagt cttgctctgt cgcccaggct ggagtgcagt
6781 ggtgcgatct cggctcactg caagotccgc ctcccaagtt aacaccattc tcctgcctca
6841 gcctcccagag tagctgggac tacaggcccc gccaccacgc ccggctaatt tcttgtatth
6901 ttagtagaga tggagtttca ccgtgttagc caggatgggc tcgatctcct gacctcatga
6961 tctgcccgcc ttggcctccc aaagtgetgg gattacaggt gtgagccacc acaccggat
7021 tacaaaaact ttttagataa ttatctgggc gacctgcctg accaaccatgg agaaaccctg
7081 tctctactaa aaatacaaaa ttagccggac atgggtggcg atgcctgtaa tcccagctac
7141 ttgggaggct gaggcaggag aatcatttga acccaggaag cagaggttgc ggtaagccga
7201 gatcatgccca ctgcactccg gtctgggagt gcactccaac aagaaggagt ttcgctcttt
7261 ttgcccaggc tggagtgcag tgggtgggatc tcagctcacc gcaacctcca cctcccgggt
7321 tcaggcgatt ctctgcctc agcctcccaa ggagtagctg ggattatagg tatgcactgt
7381 cacaccggc tacttttgta ttttttagta aggcaggttt ccaccatgtt ggcaggctg
7441 gtcttgaact caagtgatct gccctctttg gccctctctc caggaaaaaa aaaaaatcac
7501 aggtatthac aggccattcc aagtgcacaa agattgtttt tgctcatggg gacttcagta
7561 tcacagatgt taggagactt gctgctatat gtttaagaaag aagcacaaat gttgctgtag
7621 cccaaacttt tttcctcatg tttcattgca tttcagctta attggtttcc ctggtattcc
7681 tatgtattht gtggagtgtc tttaaaatca taagttggag tagaggctct tctgtgggct
7741 tcaccagact gccgagatca gggctgaaac aggtgaggac cccttctctg gagagagtct
7801 cctttctcct ctaagaggaa aggttttgag atcttttgct cattttccca ccttagcact
7861 tcttggaact taaaagaagc tggaaattht tttttttttt ttggagatgg gatctcgata
7921 tgttgccag gctggtcttg aaccccttgg ctcaagcgat cctccagcct cagcctccca
7981 aagtgtctggg attcgaggca tgagccaccg agcccaccgt gcagatggat gttttgtgc
8041 atgcttttga tgaatgcttt ctctctctca gccgtgtgcc agggcacctt caagcccctg
8101 tcaggagaag ggtcctgcca gccatgcccc gccaatagcc actctaacac cattggatca
8161 gccgtctgcc agtgcccggt cgggtacttc cgggcacgca cagacccccg ggggtgaccc
8221 tgcaccagta agtgaccagc acccagggtg agttcactgg ggaggggtca cagacctctg
8281 aggtggacc tcacatggcc ccctctctcc ctgggcttct tccctttgtc cctggcatgc
8341 ttgtccctag cccggaggaa catgtggagc ccactgtctc caaggcaaga gtccagcatg
8401 gctgctgggt cctccattgc cctctcccca ccaccgcaga gcaggctcggc ctctgcctga
8461 ctccctgggt tcctgcagcc cctcctctgg ctccgaggag cgtggtttcc cgcctgaacg
8521 gctcctcct gcacctggaa tggagtggcc cctggagtc tgggtggcga gaggacctca
8581 cctacgccct ccgctgccgg gagtggcgac ccggaggctc ctgtgcgccc tgcgggggag
8641 acctgacttt tgaccccgcc ccccgggacc tgggtggagc ctgggtgggt gttcgagggc
8701 tacgtctga cttcacctat acctttgagg tctactgcatt gaacggggta tcctccttag
8761 ccacggggcc cgtcccattt gagcctgtca atgtcaccac tgaccgagag ggtgagactt
8821 gggggctggg ggggtggtg gtctggcggg agagatgtca ctgagggcct gaaggggaga
8881 ggcaggggct gtgaagttgg gtaccccgga agtgtagagg gctaaggctt tgggggcaag
8941 aggcagaaag agggcaatgg ctgggcgagc tggctcacgc ctgtaatccc agcactttca
9001 gaggtcgaga caggcggtat acttgagccc tggagttcaa gaccagcctg ggtaacatag
9061 gaagatctct ctacaaaaaa taaaaatatt agccaggcga ggtggtgcat gcctgtggct
9121 ccagctactc aagaggctga ggcaggagga ttgcttgagc ccaggagtgc gaggctgcag
9181 tgagctatga tcgcaccgct gcctgccagc ctgggtgaca gagcagtgtg agatcctctc
9241 tcaaaataaa tgaataagaa agagaggggt aggagctcgt aaagctgggc tggagagtta
9301 agtacaggaa ggccccagc gggactgggg ccagagagaa tcagaaggaa ttctcgaaac
9361 agccaggggg aaattgagac aagtgtagcc agcagaggaa gtgttggaag cctggccaac
9421 catggccagg ctgatcacaa ggtcaggagt tcaagactag ggtgggcacc tggtaatccac
9481 cccatgtcta ctaaaaataa aaaaattagc caggagggcg gaggttgagc taagctgaga
9541 ttgggaagca accagaagaa ttgcttgaa cagcacgact ccgtctcgaa aaaaaaatt
9601 ctgcgccact gcactccagc ctgggtgata gagcacgact cactgtgtct ctgctctca
9661 ttttttaagt taagggacag agctaccatg cacaagggtt cctgtgtctc agcttgagcc
9721 cagtacctcc tgcagtgtct gacatccggg tgacgcggtc ctccccagc agcttgagcc
9781 tggcctgggc tgttccccgg gcaccagtg gggctgtgct ggactacgag gtcaaatacc
9841 atgagaaggc aaggccatcc cccagcctg ggggtgggtg gcaatgggtt gtgctctcct
9901 ggctgggaca cctgggttgc aggcacctgg caggcatttg aattccagct ctgccatgga
9961 tccccgggc agccttgggt aagccccgt gccctgtctg gccctcagct cttcatctat
10021 aaaatagtt ctgtaatagt taccagagc tggacacagt ggctgaggtt gggcgaggtg
10081 gctcacgcct gtaataccaa gcactttggg aggctgaggg gggcagaatg cttgagccta

```

10141 ggagtttgag accagcctgg gcaacatggt gaaacttcat ctctataaaa aacttaaaat
10201 gggcgggcg cggtagctta cgctgtaat ccagcactt tgggaggccg aggtggcg
10261 atcacaagg caggagatc gagaccatcc tggctaaccac ggtgaaaccc catctctact
10321 aaaaatacaa aaaattagcc aggcgcggtg gcaggcgcct gtagtcccag ctactcggga
10381 ggctgaggca ggagaatggc gtgaaccagc gaggcggagc ttgcagttag ccgagatagc
10441 gccactgcag tccggcctgg gcgaaagaac aagactctgt ctccaaaaaa aaaaaaaaaa
10501 aaaaaaacg caaaaaatac ttaaaatgaa aaaaattaga ctgggcacag tggctcatgc
10561 ctgtaatccc ggcacttttg gaggcgagg tgggtagaac acctggggtg aagagttcga
10621 gaccagcctg gccacaagg tgaaatccc gtcttacta caaatagcaa aatcagctga
10681 gtgtgttggc gggccctgt aatcccagct actcaggagg ctgagacagg agaatcactg
10741 gaaccaagt gattctcgac ttgaggtcga ggctgcagtg agtcgtgtt gcaccattgc
10801 attccagcct gagaaagtga gaccttgtct taaaaaaaag gaatgatatt atgaatacag
10861 cacatggctt gcatgcgtaa gttctccaa aggcctcacc agttgcaagg caggctagt
10921 atgggagtg agggcgagg aaggaggcag gaagagcaac aggaacttg gttcccgggt
10981 gacggccacc cactacctc tccgggacag ggcgcgagg gtcccagcag cgtgcggtc
11041 ctgaagacgt cagaaaaccg ggcagagctg cgggggctga agcggggagc cagctacctg
11101 gtgcaggtag gggcgcgctc tgaggcggc tactggccct tcggccagga acatcacagc
11161 cagacccaac tggatggtga gcctgggga gggggtgagg gtgggggtg gaaagacccc
11221 caaagttcct ggggaagacc caggtctcca aagtcctatc atcttttttt tttttttttt
11281 tttttgagat ggagtcttgc tctgtccctc aggtctgagt gcagtggcac catctccgt
11341 cactgcaacc tccgcctccc ggattcaagc cattctcctg cctcagcctc ccgagtagct
11401 gggattacag ggcctgcca ccgcgctgg ccgatttttt gtatttttag tagagacggg
11461 gcttcaccgc gttggccagg ctggtctcga actcctgacc ttgtgattcg cccgctcgg
11521 cctcccggaag tgctgggatt acaggcatga gccactgcac ccggtcaaag tcttatcttc
11581 atgtccttct tctgtggat cacatggcat gccctagaga ggagagaacg taagatgtcg
11641 aaacaaaac caacagctga gttttgtgaa gtctggcctg cttcactctg tacccccagg
11701 ctggagcgca gttgctcgat caaagctcac tgcacagcca ggcacagtgg ctcaccctgt
11761 aaccccgca ctttgggagg ctgaagcagg aggatcactt gaggtcagga gttcgagacc
11821 agtctgacca gcatggtgaa accgcgtctc tactaaaaat atagaagtta gctgagcgtg
11881 gtggtgcaca cctgtaatcc cagctactcg ggaggctgag gcaggagaat cgctgaacc
11941 tgggaggtgg aggttgcatg gagctgagat tgtgccagtg cactccagcc tgggcaacag
12001 agcaagactc tgtctcaaaa aaaaaaagc tcacgcagg cttgactttt agcaacaacc
12061 tgaccctga gctcccatc cccatccaa caaatggga atatcatgaa gcttctgca
12121 gggctttgag gattggagg aacaggttat ttttaatatg ctaggccagt ggctttctt
12181 tttctttcac attttttttt ttgagacgga gtctcactct gttgccagg ctggagtgcg
12241 gtggcgcgat ctgagctcac cgcaagctcc acctcctggt ctgatctgc tgacctctg
12301 atccaccgcg ctccgcttcc cgaaatgctg ggaactgctg cgtgagccac cagcccgcc
12361 ctactttttt ctttttttta agagacacgg tcttttttat caccagct caaccaatcc
12421 ggcaccatca tagctcattg cagcctacaa ctcccgagct caaccaatcc ttcacctta
12481 gcctcccaag tagctggggc tataggcatg tgctaccgtg ctcaactaaa ttttttttta
12541 tgttttggtg agacagtttc cctatgttgc ccaggctggt ctcaaattcc tgacctcgag
12601 caatcctccc gcatcggcct cccaaagtgc tgggattaca ggcagagcc gccacacca
12661 gcattggacc agtggcttcc taaacctgt aattttctgt aatagcttta ctgaaatata
12721 gttccctgca catacaattt gctgttcaa agtgtacaat cgatgacttt tgatacttc
12781 acagaattgt gcagtcacca ccacaagttaa ttttgggaca ttttcagcac cctcaaaaga
12841 gacctatag cccttagcca tcacccccca ccagatctt tctgttgcct tagtccctgg
12901 caagcactaa cccactttct gtcttgaat cttccagtgt ggtcttttgt gactgttcac
12961 cgagcagaat gttttcaagg tttatgtatg ttgtagtata tatccgtggg ttttttggg
13021 tgtggtttgt tttttgttgg ttttggaaac aggtctctcg tctgtcacc aggctggagt
13081 gcagtgggtc aattacagct cactgcagcc tcaacctccc aggtcaagt gatcctcca
13141 cctcagcctc ccaagcagct gggactgtg gcagagcca ccatgcccag ctaattttt
13201 ttggtatttt ttgtaaagac aggtgttcc catgtttccc aggtggtct cgaactctg
13261 agctcaggca atccaccac ctcagcctcc caaagtgtg tgattacagg catgagccac
13321 tggacctggc ctgttttttg tttttgtttt gaacacacga ttttgctttg tccccaggc
13381 tggaaatgaa tggctctgat atagtgcatt gcagcctcaa actcctgggc tcaagcgatc
13441 ctccctacctc agcctcctga gtatctggga ccacacgtgc tcaccaccat gcttggctaa
13501 ttattattat tttttgatag agacggggc ttgctatgt tcccaggctg gtcttgaaca

13561 cctggcctca cacaatcctc ccacctcagt atctcagagt gctgggatta caggcatgag
13621 ccactgctcc tggccaatat ttcattttctt tttatggaga cgtaataatc agttgtatgg
13681 aaatagctga ttttggtttt tattgtatct tttggtgaac atttcaattg tatcgacttt
13741 ttggataaaa acctgaaaat gtttcacctt tagaacgttt cattgaatgg agattttttt
13801 gtggactctg gtattttatac tagaaccaaa tcaaaaccac tctggcggct gggcatgcct
13861 aggctgggtt gtagactagcc tgtccaacct ggtgaaagcc catctctact aaaaatacac
13921 aaattagccg agcatggtgg tacacaacct taatcccagc tactcaggag gctgaggcag
13981 gagaatcgca gaacccggga ggcggagatt gcagtgaagt gagattgcgc cactgcactc
14041 cagcctgggc gacagagtga gactgcgtct caaaaaaca aacaaaaaat tactctggca
14101 gtaagaaaag atttcgaaac ttcctccctt gccctgaggt acttcagagg agcctgctgg
14161 cccctggggg agagtgtgaa acccactggt tgttccctga ccttgctgc ttgtgtcctc
14221 tccctccacc tgtccctgt actggggacc tgttctcagg agatcacagt tcattgctca
14281 aagcgggggc tggggcctcc tacaggacca tcagtttctc ctgatcagca gcctttcctt
14341 ccgcagagag cgagggtgg cgggagcagc tggccctgat tgcgggcacg gcagtcgtgg
14401 gtgtggtcct ggtcctggtg gtcattgtgg tcgcagttct ctgcctcagg taagggtctct
14461 gacacccaga ggcctcctgga agcctcaggt tgatggccac ctgcctgggt gctacaggac
14521 aagcctttct ggctgtcccc agcctctttt tacttgaaat cttctccaat ccctgctcct
14581 tcccttgggtg tgtgtgcctc ataaagatgt gtgactcagt ttaccttttg ttcctttccc
14641 atcggctaca ggaagcagag caatgggaga gaagcagaat attcggacaa acacggacag
14701 tatctcatcg gacatggtgg gttgccttaa tttgatggga ataggggctt ggggcgggtg
14761 gtggtggctc ctatctataa tcccagcact ttgggaggca gagggtggca gatcacttga
14821 ggtcaggagt tcgagaccag cctggccaac atgttgaaac tccatctcta taaaaatac
14881 atcagtcagc caggcatggt ggtgggcacc tgtaatcca gctactcagg aggctgaggc
14941 agaagaatca ttttaaccgg ggaggcggag attgcagtga gccaaagatcg cgccactgcg
15001 ctccaggcct ggggtgacaga gcgagactcc atctcaggaa aaaaaaaaaa aaaaaaaaaa
15061 accacggaga caggggtttg gggctaaaag ctatgagccg agcctccgag tccagtggga
15121 gttaattccc agctgacggg gccctgcctg atttctcagg tactaaggtc tacatcgacc
15181 ccttcactta tgaagacctt aatgaggctg tgagggaatt tgcaaaagag atcgatgtct
15241 cctacgtcaa gattgaagag gtgattggtg caggtgagag ccgaaggctg cccgggcacc
15301 tgggaacgaa gcgggggtgg gcagggccac actggagcgg gagagctgat gacctctgcg
15361 tcttgttttg aaggtaggtt tggcgaggtg tgccgggggc ggctcaaggc cccagggaag
15421 aaggagagct gtgtggcaat caagacctg aagggtggct acacggagcg gcagcggcgt
15481 gagtttctga gcgaggcctc catcatgggc cagttcgagc accccaatat catccgctg
15541 gagggcgtgg tcaccaacag catgccctg atgattctca cagagttctc gggaacggc
15601 gccctggact ccttcctgcg ggtgagcacc ctccctggct tctgcgcca cccggagttc
15661 ccacttacac ccagaggcca cttgggttaa gaagccagga cagacagtgg gtcccaggctc
15721 acctcctcca gccttttctt cttgggctaa gccctggtcc tctgcctttt ctttttttta
15781 agacagagcc tcgctctgtc gccaggtcg gactgcagt gcgcgatctc ggctcattgc
15841 tgtctccacc tccagggttc aagcgattct cctgcctcag tctcccaagt agctgggtact
15901 ataggcatgc accaccatgc tgactaattt ttgtattttt agtagacaca gggtttcacc
15961 atgtaggcca ggctggtatc aaactcctga cctcaagtga tctcccacc tcagcctccc
16021 aaagtgcctg tattacaggt gtgaggcacc acgcctggcc agcctctgc ctttaatttt
16081 cctctgaggga aaggctgggc tccctgggacc ttcctttccc actgccccat acagotgaag
16141 gttgtcatte cttctttttt tttttaattt tgttttaatt gaattttttt tttttgagat
16201 ggagtttcac tcttggtgce caggccggag tgcaatggca agatcttggc tcacgcgaac
16261 ctccgcctcc caggttcaag cgattctcct gccttagcct cccagtagc tgggattata
16321 ggcagtgtgc accacgcttg actaattttt tatttttagt agagacgggg gtttctctgt
16381 gttggtcagg ctggtctcga actcccagc tcaggtgatc cgcctgcctc ggcctcccaa
16441 agtgcctggga ttacagacgt gagccacgc gcccgccaa tttttttt ttttttttaa
16501 gacagagtct cactctgtcc tctaggctgg agtgagtggt tgcattcata gctcactgta
16561 gccttgacct cctgggctca agtgatctc ccgcctcagc ctctgagta gctggaacta
16621 cactcatgta ccaccatgct cagcaaattt ttaaaattt ttgtagagac aggatctcga
16681 taggttgccc aggtggtct gaactcctgg cctcaagcga gcctccctcc tcagcctccc
16741 acagcactgg gattgcagge atgagccact gtgcctggcc tgtcattcct tcttttgaca
16801 aatatttact gagtgccttc tacgcaccgg tcatcctccc agtccccagg aataaagcta
16861 tacacacggc aaactggatt tctcctctg gggagcagag ggtctaattg ggcggggga
16921 ctgaaaatta gcaagtaaat agacaggctt tttaaaaaag taaacaatc atttcaaatg

16981 tgaaaaaaag caaacggggt ccttcattgca gatgtggcta gagaggaaag agaactgctt
17041 aatttatttg gtcactttac cagattttac tgactttttt ttttttttta actttattaa
17101 gctttttctt tttcttgaga tggagtttcc atctgtcacc caggctggag tgcagtgggtg
17161 cgttcttggtc tcaccgcaac gtccacctcc tgggttcaag tgattctcct gctcagcct
17221 cctgagtagc ttggaattgc atggcatgca ccaccatacc cagctgatgt ttgtattttt
17281 agtagagaca gggtttcato atgttgccca ggctgggtctt gaactcctgg gctcaagtga
17341 tccacccatc tcggccctcc aaagtgtctg gattacaggg atgagccacc atgctggcc
17401 taggcattct tttaaaaaaa tcaaaacatt tttctatgta gcaaaataac attgcattga
17461 acagagttat agcgattccc tagcgtcatt gaatacccag ttgattttca cgtttctcta
17521 gttgttctaa agatgtcctt cactgctgct ttattccaac caggatccag ttcaagaccg
17581 ggctttgtac ctgggtatta tatatatttt atttatttat tttagaaaca aggtcttgcc
17641 ctttcgcccc gtttagagtg cagtgggtgca atcatagctc actgcagcct ccaaactcct
17701 tggctcaggt gatcctcctg cctcagcctc ctgggtagct ggaactacag gtgcacacca
17761 ccacacctgg ctaattttta aattttttac ggagatgggg gtctcgctat gttgcccagg
17821 ctggtctcaa actcctggac tcaagcgatc ctccctcctt aacctctcaa agtgcctggga
17881 ttacaggcgt gagccaccac gctcgtgat tattatattt tcagacctct ctaaatcttg
17941 agcagttcct catgatgaca ctgacacact gaagggttag gtccctgtc cgctgaatg
18001 tcttgatttc tggatttatg aaattcttct tatgggatca tttagcttgt ctctctgtat
18061 ttctgttaag agaagctcta tctgatgtgg gggttttttg gttttgttg tttgtttttt
18121 gagatggagt cctgctgtcg ccaggctgg agtgcagtgg cacaatctcg gctcactgca
18181 acctccgct cctgggttca agagattctt ctgctcagc ctctgagta gctgggacta
18241 caggcgagtg ccaccatgcc cagctaattt ttgtattttt agtagagaca gggtttcacc
18301 atattggcca ggatgggtct gaacttctga cctcgtgatc tgcccaccac ctcagcctcc
18361 cacagttcct tgcatcagg catgagccac tatgccggc taatttttgt atttttagta
18421 gagacagggc ttgcctatgt tggccaggct gatctgaaac ccctggcctc aagccatcca
18481 cctccttgg cctcccaaag tgctgggatt aaacgcgtga gccaccgtgc ctggtcgaag
18541 agacagaaag ggtcttaaag gttcagtac acacacctgt aatcccagca ctttgggaag
18601 ctgaggctgg tggatcactc gaggccagga gttagagatc accctgggca acatggtgaa
18661 acccctgctc tacacaaaat acaaaaatgg gcagagcatg atgggtcata tctgtagtc
18721 cagctactcg ggaggctgag gcgggaggt cacttaagcc tgggagatcg agtcttagt
18781 gagccatcat tgccactact cactccagcc tgggcgatcc catctcttaa aaagagagag
18841 agatgggaag accagcacag gtgaaactgg tgaacagagg agagatggta gatgctgcat
18901 tgggcagtgt gacgggaacc cgctggagg ctttggcagg agagtagttt aagaggatcc
18961 cagctgggca cagtgggtca cacttgtgat ccagcactt ggggaggccg gggcaggtgg
19021 atcacttgag gtcaggagtt cgagaccagc ctggccaaca tgggtgaaacc ctgtctgtac
19081 taaaaataca aaaaccagcc aggcattggt gtgcaccct gtaatcccag tcttagtcc
19141 gactaagaca ggagaatcgc ttgaaactga gaggcagagg ttgcagtga ccaagatcac
19201 gccactttac tccagcctgg gcagtagagc gagactccat ctcaaaaaaa taaataaata
19261 aaaagacctc tttgctgggt gctaggagc aagagcagga gctgggagag gcctgcagca
19321 gaagcctgtt gccagcatcc aggcctggtg gtgaaggga ggggttggat ttgggacatg
19381 tcttggaagc atcaccagca gaacttgct atggattgga agtggctggt gaggagaaa
19441 aggggggtcaa aggaaactct gaggtctata ccctgaccat ctggcaagtg gtggtgttgc
19501 cacaaactga gcggggagta gggcaggtgc aggtctggag gatggattca aaattcagtt
19561 tttggagtct atgtccctgg ttctgtagg ctgcagatgg tctgccaat cttagcgga
19621 cccagaatac gggatttgtt tactgtctgt gacttgttg tttccctggt gagagcaaac
19681 tctttaaagg tcaagggttg gcttcagacc ttgggttttg caccgatcat tggctactat
19741 gcagttcctc actcttctct tgcaaatcca tacacagcta gtccaagaga gctgaacagc
19801 tttgtggttg gatcagcacc aatgtatctc cacctgtaga cgggttgcct aggtgaacta
19861 tgctgtaat cccagcacct tgggaggcca aggtgggaag attgcttgag gccaggagtt
19921 ggagacaagc ctgggaaaca cagtgcagcc ccatactac caaaaaaac cctttgtttt
19981 aattagccag gtgcagtgg gtgcacctat agtccagct actaaggagg ctgaggcaga
20041 aggatcattt gagccagga gtttaaggct gcggtgaacc atgatcgtgc cactgcactc
20101 caacctgggg gaaagaaaga gacctgtct ctaaaaaac taaaaaacag aaaagcattt
20161 gttgagtatt tctgggtat aaagcagtgt accaggttaa atggaaggaa aggttgaaat
20221 aatttttcaa ctcataatcc gattgggaga gactgaatgc ttaccattga agcaggaacc
20281 attgtaagca atgtgttgtg atactgtagc aagagctgag aaaacttggg aaaagagaaa
20341 ggaggaaggg tcacctgagg gaggttgggg gcttgcccta caggtagatt gtgagggtggg

20401 tctggaagt acagatgcag tttaggaagt ggacgggagg ctgggtacgg tgactcaaca
20461 tctgtaatcc cagtgttttg ggagaccag gcggaaggat cgcttcaggc caggagttaa
20521 agaccagcct gggcaacata gtgggaacct atctctacta aaaattaaaa aattatccag
20581 gcataatggc acatgcctat tgttccagct actcaggagg cttgcctgag cccaggaggt
20641 tgaggctgca gtgagctatg atggcaccac tgcactccag cctggggcagc agaacaagac
20701 cctgtctcta aaaaaaaaaag atgtggatgg gagggggaac ggtgggtggg ctgtccctcac
20761 caagccccc caactatctgc tctccagcta aacgacggac agttcacagt catccagctc
20821 gtgggcatgc tggggggcat cgccctgggc atgcgggtacc ttgccgagat gagctacgtc
20881 caccgagacc tggctgtctg caacatccta gtcaacagca acctcgtctg caaagtgtct
20941 gactttggcc tttcccgatt cctggaggag aactcttccg atccaccta caccagctcc
21001 ctgtaaatcc tgggggtaat actgggtgtg agcttcttag ggccagggtg gcagggcagg
21061 ttggaaagggt gggaggctga ggggttggca gccctgtctc agggagagga tacaggagca
21121 ggctgtgggt ggggggacag tcagctccag gaagccgact tccagatgtc taggaaaata
21181 acagttggat aacctgggca acatagcaag accccatctc taaaaaaaaa ttaaaagatt
21241 agccaggcgc agtggcatgc acctgtagt cagctactt gggagggtga ggcaggagga
21301 ttgcttaagc ccaggagtg gaggtgcag tgagctatga atgtgccact gtactcaga
21361 ctgggagaca gagcaagacc ctgtctcaaa agaacagtgg ccagggtggg ttggtcacgc
21421 ctgtaaatcc agcactttgg gaggtgagg caggaggatc gcctgaggtc aggagttcga
21481 gaccagcctg gccaacatgg gaaaaccctg tcgctactaa aaatacaaaa ttagctgagg
21541 gtgggtgtac acgctgttaa tccgagctac tcaggaggct gaggtaggag aaccagttga
21601 acccgaggag cggagtttca gtgagccaag atcgccacc tgcactccaa cctgggcaaa
21661 cagagttgga gagtaggagg cttggggcct gagctagggg gaaaaagcag aggcaggtg
21721 gggactgggg ggcagtgctg tgggtctggt gactccctca gtgagtcctc cagctcacct
21781 tttctccttt ttctgcagg aggaagatt cgccagtgat gcctggagtt acgggattgt gatgtgggag
21841 gccttcggga agttcacttc cgcagtgat gacatgagca atcaggacgt aagtgtccc
21901 tggatgtcat ttggggagag gccgtactgg gacatgagca atcaggacgt aagtgtccc
21961 tggctcctacc aagctttcct cgagtggtct ctcacctggg atttgggggtg aagggtgggt
22021 tcccagagag tcatcactgc tgggttcttg agaccatgga gatgacaaaa aggagaattg
22081 atctttgtat caaagagttg agatacaggg ccaggcctag tggctcaagc ctgtaatccc
22141 agcactttgg gaggccaaag tgggcagatc acctaaaggtt aggagttcaa gaccagcctg
22201 gccaacatgg tgaaaccccg tctctaaaaa atatacaaaa attagccagc attaggggc
22261 ggggtgcctg actcccagct actcaggag ctgagacagg ataactcgctt gaaccaggga
22321 acagaggttg cagtgaagct agatcacgcc attgttttcc agcctgggca actgagcgag
22381 actctgtctt aataaataaa taaagaggtt gggtagagca tatttgggtc gcagaaggat
22441 gcagagatgg agggcagggt tgagaggtaa catgtctgta tcatagccca agagctgctg
22501 gggccttcag ccacagagag cttcaactcc ggctaggagg attcctggat ctgttatttt
22561 ttgggggggt gtgggtccta tccctaccatc ttccaagtca ccatttcttg ggctgttag
22621 catctttgct tttcctggac agcctcacc agagcttctt cccctcttc cagggtatca
22681 atgccattga acaggactac cggctgcccc cgccccaga ctgtccacc tccctccacc
22741 agctcatgct ggactgttg cagaaagacc ggaatgccc gccccgctt cccagggtg
22801 tcagcgcctt ggacaagatg atccggaacc ccgccagcct caaaatcgtg gcccgggaga
22861 atggcggtg aggactgcag agaatgggct ctccttccc ctctctgccc cactccttg
22921 cccagaagtg tccgttcatt ggtgttgggt gggaggccct ctgtccgct ctgcaaggct
22981 gggttccacc tccctccccg gacctgggct tggtagctc cactcctcc catccttgc
23041 ccctagggcc tcacaccctc tccgtgacca gcggcagcct cactactcag cttttggctc
23101 tgtgggcgag tggcttcggg ccatcaaaat gggaagatac gaagaaagtt tcgcagcgc
23161 tggctttggc tccctcgagc tggtagacca gatctctgct gagtaagcag tggcaggagc
23221 tggagtgggg ctgggagagc ggggcagctg gactcaggcc cacggggtct ccaggggctt
23281 ttggggtcag ctccgggtgc caatgtgtc ttcttgact gcgctcatgc catgcttaga
23341 agggcccccag aggagcagtc acagcccat ggagctgagg acccaaggac tctttggggc
23401 cagcctgccc gcctcacctc ctccctgcat cacagccctg ggccatcgcc cttccgctc
23461 tcacttctag ctatctttgt gcactctatc gcattccagg cccggctctc acggtaacaa
23521 tgtgtcaact cgggttctct ttttccaaac ataaaaggag aagattggg taggttttg
23581 agatcctctt cagcttttat gtgaaatgggt tttatgattc cttgctccc aaaggctg
23641 tatcccaact tggcctttgt ctgctactcc ccccttctgc cttcccgctt ctctcccaag
23701 atctcctctc accccagggt gaataacaga aatagaagga atagaaatct gaaggccggg
23761 catggtggct catgcctgta atgccagcac tttgggaggc cgagggtggg agatcacttg

23821 aggttaggag ttcgagacca ttgtggacaa cttggtgaaa ctttatgtct actaaaaata
23881 caaaaattag ctgggcatgg tgggtgctgc ctgtaatacc agctactgag gaggctgagg
23941 caggagaatc gcttgaaccc gggaggtgga gggtgcagtg agccgagatc gcaccactgc
24001 actccagcct ggatgacaga gtgaaattcc atctcaaaaa aaaaaaaaaa aaaaaaaaaag
24061 aaatgtgaag gccaggtggt ggctcacgcc tghtaatctca gcactttggg aggctcaggt
24121 ggaccgattg cttgagccca ggagtttgag agcagcctgg ccaaaatagc aaaaccccat
24181 ctctacaaaa caaaaacaaa aaaattagct gggcatggtg gtgctgctgt gtggtcccag
24241 ctactcagga ggctagagcc agagggtctc agggcagtet gcccctgccc cacggggcct
24301 gggcacatcc ctccctaatt cttcccagcc tctctctgac ccagggggcc tctctccct
24361 tttttccctt tatctcagcc tccagccatc agcaacctcc tcttctctc caccagctc
24421 ttctctccc acttcggcct tttctttctc acactccatt tccctctacg gcaatctgtg
24481 cagcctcttc cccagctctc attttgcggg cttttctctc ttttctttcc ttccctggca
24541 cccaagccaa aggccttgcc tctggcctcc agccctaccc ccttctgctg ttgcacagaa
24601 ggatggctgc ccagctctta aaaaaactgc ccgggaactg ttgacatctg ttctccctcc
24661 cccgctggct tttctgattg gcttacaatc ctgaggctag gaccgtctca ggagccaaga
24721 gaggagagcg gccacaggga acctagggtc tcaccaagct ctcttctctc tctgcaggga
24781 cctgctccga atcggagtca ctctggcggg acaccagaag aaaatcttgg ccagtgtcca
24841 gcacatgaag tcccaggcca agccgggaac cccgggtggg acaggaggac cggcccgcga
24901 gtactgacct gcaggaaact cccaccccag ggacaccgcc tccccatttt ccggggcaga
24961 gtggggactc acagaggccc ccagccctgt gcccgcctgg attgcacttt gagcccgagg
25021 ggtgaggagt tggcaatttg gagagacagg atttgggggt tctgccataa taggagggga
25081 aaatcacccc ccagccacct cggggaaact cagaccaagg gtgaggggag ctttccctca
25141 ggactgggtg tgaccagagg aaaaggaagt gcccacatc tcccagctc cccaggtgcc
25201 cccctcacct tgatgggtgc gttcccgcag accaaagaga gtgtgactcc cttgccagct
25261 ccagagtggg ggggctgtcc cagggggcaa gaaggggtgt cagggcccag tgacaaaatc
25321 attgggggtt gtagtcccaa cttgctgctg tcaccaccaa actcaatcat ttttttccct
25381 tghtaaatgcc cctccccag ctgctgcctt catattgaag gtttttgagt tttgtttttg
25441 gtcttaattt ttctccccgt tccctttttg tttcttcgtt ttgtttttct accgtccctg
25501 tcataacttt gtgttgagg gaacctgttt cactatggcc tcctttgccc aagttgaaac
25561 aggggcccac catcatgtct gtttccagaa cagtgccttg gtcaccccac atccccggac
25621 ccgcctggg accccaagc tgtgtcctat gaaggggtgt ggggtgaggt agtgaaaagg
25681 gcggtagttg gtggtggaac ccagaaacgg acgcccgtgc ttggaggggt tcttaaatta
25741 tatttaaaaa agtaactttt tgtataaata aaagaaaatg ggacgtgtcc cagctccagg
25801 ggtgatgggg gtgatggact agatttctaa ggagagtggg gctgggtagg gagggctttg
25861 tggctgaccg agagggtgtca gaggtctgga ggctgcaggg ctgtaggggc tggaacttgg
25921 ttatcagccc cagggtatgt ttgaggtggt ggggtggggg ccgagcgaga tgaatcatte
25981 gcagctgctt ctaacgtctc

FIGURE 62. EphB4, mRNA.

```
1  ctcggccocgg  cggcgcgagc  agagccactc  cagggagggg  gggagaccgc  gagcggccgg
61  ctcagccccc  gccacccggg  gcgggacccc  gagggccccc  agggacccca  actccagcca
121 cgtcttctgt  cgcgcccgc  cggcgcgggc  actgccagca  cgctccgggc  ccgcccggc
181 cgcgcgcggc  acagacgcgg  ggccacactt  ggcgcgcggc  ccgggtgccc  cgcacgctcg
241 catgggccc  cgctgagggc  cccgacgagg  agtcccgcgc  ggagtatcgg  cgtccacccg
301 cccagggaga  gtcagacctg  gggggcgagg  ggcccccaa  actcagttcg  gatcctacce
361 gagtgagggc  gcgccatgga  gctccgggtg  ctgctctgct  gggcttcgtt  ggccgcagct
421 ttggaagaga  ccctgctgaa  cacaaaattg  gaaactgctg  atctgaagtg  ggtgacattc
481 cctcaggtgg  acgggcagtg  ggaggaactg  agcggcctgg  atgaggaaca  gcacagcgtg
541 cgcacctacg  aagtgtgtga  cgtgcagcgt  gccccggggc  agggccactg  gcttcgcaca
601 ggttggggtc  cacggcgggg  cgccgtccac  gtgtacgcca  cgctgcgctt  caccatgctc
661 gagtgcctgt  ccctgcctcg  ggctggggcg  gggcacggcc  ctcacgccag  cctggatgga
721 tatgagagc  atgcggacac  ggccacggcc  ctcacgccag  agcgccctgg  ggcgcaggcc
781 atcaaggtgg  acacgggtgg  cgcggagcat  ctcacccgga  agcgccctgg  ggcgcaggcc
841 accgggaagg  tgaatgtcaa  gacgctgcgt  ctgggaccgc  tcagcaaggc  tggcttctac
901 ctggccttcc  aggaccaggg  tgccctgcag  gccctgctat  ccctgcacct  cttctacaaa
961 aagtgcgccc  agctgactgt  gaacctgact  cgattcccgg  agactgtgoc  tggggagctg
1021 gttgtgccc  tggccggtag  ctgctggtg  gatgccgtcc  ccgcccctgg  ccccgagccc
1081 agcctctact  gccgtgagga  tggccagtg  ggcgaacagc  cggtcacggg  ctgcagctgt
1141 gctccgggg  tcgaggcagc  tgaggggaa  accaagtgc  gagcctgtgc  ccagggcacc
1201 ttcaagcccc  tgtcaggaga  agggctctgc  cagccatgcc  cagccaatag  ccactctaac
1261 accattggat  cagccgtctg  ccagtgcgc  gtcgggtact  tccgggcacg  cacagacccc
1321 cggggtgcac  cctgcaccac  ccctccttcg  gctccgcgga  gcgtgggttc  ccgctgaac
1381 ggctcctccc  tgcacctgga  atggagtgc  cccctggagt  ctgggtggcg  agaggacctc
1441 acctacgccc  tccgctgccc  ggagtgcga  cccggaggct  cctgtgoccc  ctgcccggga
1501 gacctgactt  ttgaccccg  ccccccggac  ctgggtggag  cctgggtggg  ggttcgaggg
1561 ctacgtcctg  acttcacct  tacctttgag  gtcactgcac  tgaaoggggt  atctcctta
1621 gccacggggc  ccgtccatt  tgagcctgtc  aatgtacca  ctgaccgaga  ggtacctcct
1681 gcagtgtctg  acatccgggt  gacgcgggtc  tcacccagca  gcttgagcct  ggcctgggct
1741 gttccccggg  caccagtg  ggctgtgctg  gactacgagg  tcaataacca  tgagaagggc
1801 gccgagggtc  ccagcagcgt  gcggttcctg  aagacgtcag  aaaaccgggc  agagctgcgg
1861 gggctgaagc  ggggagccag  ctacctggtg  caggtacggg  cgcgctctga  ggcgggctac
1921 gggcccttcg  gccaggaaca  tcacagccag  acccaactgg  atgagagcga  gggctggcgg
1981 gaggagctgg  ccctgattgc  gggcacggca  gtcgtgggtg  tggctcgtgt  cctgggtgtc
2041 attgtggctg  cagttctctg  cctcaggaag  cagagcaatg  ggagagaagc  agaataattc
2101 gacaaacacg  gacagtatct  catcggacat  ggtactaagg  tctacatoga  ccccttact
2161 tatgaagacc  ctaatgaggc  tgtgagggaa  tttgcaaaag  agatcgatgt  ctctacgtc
2221 aagattgaag  aggtgattgg  tgcaggtgag  tttggcgagg  tgtgccgggg  gcggtcaag
2281 gcccagggga  agaaggagag  ctgtgtggca  atcaagacc  tgaagggtgg  ctacacggag
2341 cggcagcggc  gtgagtttct  gagcgaggcc  tccatcatgg  gccagttcga  gcacccaat
2401 atcatccgcc  tggagggcgt  ggtcaccaac  agcatgcccg  tcatgattct  cacagagttc
2461 atggagaacg  gcgcccgtga  ctccctcctg  cggctaaaac  acggacagtt  cacagtcatc
2521 cagctcgtgg  gcatgctgcg  gggcatcgcc  tcgggcatgc  ggtaccttgc  cgagatgagc
2581 tacgtccacc  gagacctggc  tgctcgcaac  atcctagtca  acagcaacct  cgtctgcaaa
2641 gtgtctgact  ttggcctttc  ccgattcctg  gaggagaact  ctccgatcc  cacctacacg
2701 agctccctgg  gaggaaagat  tcccatccga  tggactgccc  cggaggccat  tgcttcggg
2761 aagttcaact  ccgccagtga  tgctggagt  tacgggattg  tgatgtggga  ggtgatgtca
2821 tttggggaga  ggccgtactg  ggacatgagc  aatcaggacg  tgatcaatgc  tactgaacag
2881 gactaccggc  tgcccccgcc  cccagactgt  cccacctccc  tccaccagct  catgctggac
2941 tgttggcaga  aagaccggaa  tgccccggcc  cgcttcccc  aggtggtcag  cgccctggac
3001 aagatgatcc  ggaaccccg  cagcctcaaa  atcgtggccc  gggagaatgg  cggggcctca
3061 caccctctcc  tggaccagcg  gcagcctcac  tactcagctt  ttggctctgt  gggcgagtg
3121 cttcgggcca  tcaaaatggg  aagatacgaa  gaaagtctcg  cagccgctgg  ctttggctcc
3181 ttcgagctgg  tcagccagat  ctctgctgag  gacctgctcc  gaatcggagt  cactctggcg
```



```
3241 ggacaccaga agaaaatcct ggccagtgtc cagcacatga agtcccaggc caagccggga
3301 accccgggtg ggacaggagg accggccccg cagtactgac ctgcaggaac tcccccccc
3361 agggacaccg cctccccatt ttccggggca gagtggggac tcacagaggc cccagccct
3421 gtgccccgct ggattgcact ttgagcccgt ggggtgagga gttggcaatt tggagagaca
3481 ggatttgggg gttctgccat aataggagg gaaaatcacc cccagccac ctccgggaac
3541 tccagaccaa ggggtgaggc gcctttccct caggactggg tgtgaccaga ggaaaaggaa
3601 gtgccaaca tctcccagcc tccccagggt ccccccctac cttgatgggt gcgttccgc
3661 agaccaaaga gagtgtgact cccttgccag ctccagagt ggggggctgt cccagggggc
3721 aagaaggggt gtcaggggcc agtgacaaaa tcattggggg ttgtagtccc aacttgctgc
3781 tgtcaccacc aaactcaatc atttttttcc cttgtaaatg ccctcccc agctgctgc
3841 ttcataattga aggtttttga gttttgtttt tggcttaat ttttctcccc gtccctttt
3901 tgtttcttcg ttttgtttt ctaccgtcct tgtcataact ttgtgttgga gggaacctgt
3961 ttcactatgg cctcctttgc ccaagttgaa acagggggcc atcatcatgt ctgtttccag
4021 aacagtgcct tggatcatccc acatccccgg accccgcctg ggaccccaa gctgtgtcct
4081 atgaaggggt gtggggtgag gtagtgaaaa gggcggtagt tgggtgtgga accagaaac
4141 ggacgccggt gcttggagg gttcttaaat tatatttaa aaagtaact tttgtataaa
4201 taaaagaaaa tgggacgtgt cccagctcca ggggt
```

FIGURE 63. EphrinB2 Gene

```

1  ggcgcctcgga gctgcctgcg ggcgcacgcc gtcttccccg ccagttctgcc ccggaggatt
61  ggggggtccca gctgcctgcc cgtcagtcgc ttcttggccc ggagtgccgc gagctgggag
121  tggcttccgc atggtctgtga gaagggaactc cgtgttggag tactctggg gtgtttgat
181  ggttttatgc agaactcgga tttccaaatc gatagtttca gaggctatct attggaatc
241  ctccgaactcc aagtaagtgg cgtccgcgat cccctatgt ccccgccccg gggtcgcgcg
301  cgccgtcccg gcgggaggag gggtcagtc gcggggcctc ggagcctgtt tctggaacct
361  cggttccccg tccccacccc ccaacccccg ccccatctca ctagggtggag actcctcgct
421  cggttttcca acccgagccc cgctggaacg gacggtctct ccgcctttcc tccccgaac
481  gctcccaggc gctaaaagct actatcggt cgggtgtcaa gtccgggaag gtgtccgatg
541  cggatacctg accctctcct gtttccgagg acgaaggaca tggccacaat ctagggtggc
601  cggcacgcgg ggactggtgg gctctggaga gaggcggaga tgctgcattc gcggggagcg
661  cgggcggcgt ggtccggggc ccgcgggcgg gcgaccgggg tggcaggacg ctggcagoga
721  agcgcgttct ggagagggga gcctggagtc gctacgctgc ccgcagagcc ctggagccgg
781  ggcgccttgg caccgcgccg ccagcccgag ggtgcgcggg gagctcgctt gcttcgcagg
841  agaactcggg cgtcgagccc tttcctccgc gccggggaga cgggccttag gcttctccct
901  gagggcccg cgcacctcgg cctcccgctt cgttcataag ccggtagccc cggagtatgc
961  ggtctcgatg gccgacctga ttgtaatgca cttcctataa aagcctaggg cctggcccag
1021  tcgacactgc tccgtgaagc ttctccctcg ggaccctggt aggaatggga tccctaggat
1081  cagatttgct cttaccggac tctacagccg ggagcgagcc aggccttggt gagagtaact
1141  ttcagtttgg gccaccagag tgcattcaga atttagaaaa tccatccat ccctaaatct
1201  gtgtggtcat aactcgtagt catctgggta ttcagtactg tgcctccct tatttcgaat
1261  cacagccaaa acatatttta cagaatcttg gaattgtagt ctcgggaaac ttggagaaga
1321  agtatgcaga cattagctgg tttctggaga aaacgtttga gatcagaagc aaaatcaatg
1381  gcctaattga agttgagcaa gttggcgctg gttttaggag aaaagaaatg ggggattgat
1441  ttagaaatca cgtcttaaaag gagtgtgttc attctcttaa aagtgtcaaa tttcaaattc
1501  actaacatgt taaccaagaa tcccttcagt aaaagggcga aaacgtcggt tacaatcgg
1561  tttaaacaaa tgtttgatg atgctagaag gcactttcaa caccgctcat acggagaagt
1621  tacttagctc tgcctccttc catgtagtct gctcttgcat ggattatatt tttaatgtaa
1681  attgttgat ttgctgatga agtactggcg gcggcatctt tgcctcgatg ccggctcggg
1741  aggcgcagag tggtgcccga aggagccggg ctaggacctc gcgcagcagc gggctccgga
1801  gtccgggaga ggcgggcggg cgggcgaggg ggtcgcgggg agcccgcgcc gccgctgccc
1861  gcccggtgcc tccagaggtc actcttccat gcggaatcgc gcagcgccag gcctcgcccc
1921  tccccagggc cgcctgctcc agccactctg cactttcact gaccggttct ctttgaggct
1981  gttttttttt ttcttatgag gatttaatat ttctgtttta atctagttga aagcaattcc
2041  gttagcctct tcagcgttta gttcggtgtg tgcattctta tctttgcgct atattaacta
2101  ttagtttggt tgcattccgt aggagaatta gaaataccta gttgggagaa aaagaaaagt
2161  agaacaatag ttatttcaac ctaaggttta gacgttaata acttcttttt gtaattgttc
2221  gagatggggg gtcctggggg gaggtgacag gtaactacca ctccccccc ccattctgat
2281  gatgaagatg agtctgtctt tccagctatg tccagacctg cgagggccct gcgtttctgg
2341  aagcctgccg tttgcgcggg tgaggttget gctgctgtct tgcctccac agcagcattt
2401  cttttaaaaa tctcctgata acggcctgcc tggatgactg gataatgtgt gcctggaaaa
2461  ggtctccctt gcagctgaat gctagctcca gagatcagaa agatttcttc ctgtaggagc
2521  cataggaaag agtctctctt aagtttttga gaatgcatac aacccctga tgacaggggg
2581  tcgctttcct tggggaagtt ttatatattt ttccagagga aagtttgaat cggtaaatat
2641  gatgtggcag gaagtaatc aaatgcattg aagtttcaca tcagttccta tgaactgtgg
2701  aacaattcat ttgtaatgaa gccgccatca gtaattagat ttgtttcatt cagaggtcag
2761  ctttttttagc aggtggtcga cacagggagc atgcagcagc tgtttggata cagggtccag
2821  aaaacccttt gtaaattcag cgtctccgta actactttta tcacattgtc ggctctcccc
2881  tccctgactg tatgtaataa tggaaagatg tccctgcgtg tgaaacagta gctgccctgt
2941  taggttatcc acattgcttt gatacgttct ggtagagttg ggtccggtgt agccattttg
3001  gttgttttaa gttttgggtt ttttttttgt ttttttttaa ttcagcagag aacagtaatg
3061  cctagcttcc gtttttaact taacacttca gtagaacatt ttctccaag agggagattt
3121  tggcctaagt aaagtgtgg gctctttttt aaaaaaaat taattttact ttaattgtgag
3181  caaatctgta ttggtatggg gttctgcaat gcattacact gactttgaaa atttcgagta
3241  ctaatgcctt atgtctgggg ttaccattcc ctgtgcatac catactagtt agttaacata

```

3301 gcatttttgct tttcccatgt aatttttttcc ctatataata ctggattcct gataactaatt
3361 gacttgatac aaaaagaatgg ctggatgata tccagataac gtataataca tgggcttcac
3421 cacaatcagg ctctgaataa atacagacct gtcagagatt gataaaataa actacaatgg
3481 atagtgtctgt ttaaacagtc cattcaataa catatataag ccagcctgcc ttccattgtg
3541 tctgaaattc ttatttttgt aggtaaacaa atgcacattc agcactgatt gaatagcccc
3601 ttgaactatg ctccacagtt tgcgtttggg ttaatcttgt cggttttaat atagagagaa
3661 aaaagctcaa agcaccaggg gtggaattgt tagtgctttc acatccacat tccctcacatt
3721 ttgtcaggat gataaactgt aggtaatgga ctgtcgttgt tctgcaggac aactgagcca
3781 ggcagagcac aaagactaag ctaaagcgat acctcacaac atgcttggtg gccttctttt
3841 cagatgagaa tttatttgag aatcatgtgt ctagggactg cacatcttaa cctcaacagt
3901 tacagcttca agccccagaa acaggagctg gaggttaaga tgatttgcta agcacctggg
3961 tctaaatctt ttacaaagca taagctgttg acgctggttc tgccgacgca aagacatgca
4021 gatgactcca acatttccag aggcttctga cttaagctaa agtgtgtgga cagggtgaatt
4081 cgccatgggc ctggagacca gcttgctaaa aactatgtgt ttgaatgggt cctccagaca
4141 gagtgcagctg aagaacaatt ggtggattta tattaataacc tcttgcctgt aaacttactg
4201 aggtgcattc ttcggttggg ggatcagtga gataattgcc ttcagatgga cattgcaact
4261 ggagcaacta aatccttgcgt gtcttccctt cctctgaaat cctccaggta gctccgaga
4321 gcttcagtat gacaccaaac ttccggcgac gtttttagagt gcgttcacct aatgggaaac
4381 tattcgagat cccagcgtga ctgcagtaat gcgtcatagg aatgggagtg gcaggggaaa
4441 aggaaataca gattgttagac cctaataaaa aaatttttag gaaagatat tctttaacgt
4501 tttatgagaa cttcattctt aaaatactta attgcaaatt agacaaatag aagtgtctct
4561 ctaaggaagg tgattaaact ggtcctccta tcagcctaatt ctctgcctgc ctttgcctgt
4621 gacataaaga acctgttttt caggtcactt aatatacatc tacatagatt tgcttatgag
4681 ctcacccttt gtgtagcga gttagaccct aaagaggagt gctcaactgt ttaaaatat
4741 ttgattaaaa tatgcagaac ccatagaact ataagcttct agtcaggaat agtctttt
4801 agggaaacagc tcccccttc tttttaaggg gggaattaga aggaggctgg gggaggaata
4861 taagaacagc aaagaaggaa ggatagcaaa tgggacatgt tccgaacagc ttggaaaaac
4921 tcctgtgggt tcattgtctc tataaagcca aagaatacaa agacataagc aattcagccc
4981 ttctcccatg atggaagatg taaaccgttg acatgcctcc cctgtttaac ttgtttaatt
5041 ctcatthtaa attcagcacg atactagccg tgtgaactct gaagatttct ttagtaatcc
5101 attttgtagt tccgaatcaa aaacaaagt aaagggtctg acacaatttg cttttatttt
5161 taggcaaatc aacctgggtc atagttaata aggggattac aactcagact aggtctttac
5221 agatgtgatg taaatcaagg gcagagtata aagaaactga tcccttttga ttgaagtata
5281 gtaaaaaggc atagagaaac tagcagcagt aatctgattg tatggcaata aaaccaccat
5341 tttctgtctt tcagataaaa ataatgtggg aaatccatgc agttcataag atgtaaaggc
5401 agataaaagg tgaagccatg gcaacatata gattagcttg atgttagaaa tgacacgtct
5461 ctgaaaaggc cgcgggacga aggcccttgc ctccaggctg ttgggcatta tgtgagaacc
5521 acacagactt ggaactggg attaggaagt atgaaaagct tacttgtggg ctgggtaggc
5581 tgaggcagta aagaaaagct gctcagttct tgctcattgg tgggtggataa ttggcaaaag
5641 gttagatttca ttgactgcct tttttataga ttgagattgg ggctgattaa aacttcagat
5701 cactgcagtt gttagggcct gggagatttt cctttttaac tccctgccta acagcagcag
5761 ccgttctgta ggattaactg cacttcgcgg tcgttgccct aatctatttg ggcttcaggc
5821 agggacatgc tgggaaggaa cagagaccag aggggatagg tagggctggg gttatctgaa
5881 aagaaaacag agaccttttg atttcagcca tcttttcaga cccagctccc tctccgctg
5941 catgggagaa gcaaaggtaa acaggacaca ttgtccctct cctcagcca cagagctctt
6001 ctgtgagttt tgtctttccc accctggaaa aaaagataaa atacaatttt taaaagggga
6061 gggaggaatt tagttttaat tcaaagtgt agtaatccaa tatgccaaaa gcagtgggct
6121 ctacctagat gtaattttac tcgtaaatgt gactcttaaa ctttgagttg aatggggcag
6181 gctgttagag gtggtgtaaa ttacaggatt ataaaaatgt tagtgctgcc cagccttaaa
6241 gtcaaaaaca gaaaaatctc tgtgctgttg agtcttcccg cctctctccc tgaacaacct
6301 tgtaagtaag ctgacttttt gtttttgctt tccatacttt ccatttcagc cattaacaaa
6361 aataagccat tgaaaccagc attgggttcc atgcagagt acatccgcaa tccgggtcaag
6421 ccagaaggaa gatcttgctc gattgccccc tatttggcat tacaggaaag tctccacact
6481 ttggaagagt ctgaactctc aagacattga aaatgccaaa ggctgcaaac acctgtgtc
6541 tttcttgatg gagtgcattc tgggtgtgtt taaaaagggg aattcagtg cgtttttttg
6601 ttgtgtgtgt tgtttttttt ttttaaaagag cagcataggg cccttctaga ctcttgatt
6661 ctgtgtctga caaaaatggt cattaaatga gcaatattat aatttagacc catttactg

6721 attttgttcc aaattctcaa ctgacttgag catctgtttg gggctgtaga tacattgccc
6781 ttgttgactg tttttctcgt ttctatggga attactgtag ccattaactat gtactgttca
6841 tagactcaaa acatttttaa agtattgcat ataggctggc catatccagt gcctgttact
6901 ttaccttctt tttctaactt aatgcagcag tctgtattaa cagatccatt tcatttgtct
6961 agcttcatca gagagaggct acccctgat ttacaggctg ctcacatcca agcaccttgc
7021 attctacact tgacagtgat tgctaattggc ccattcaact aaagtatttg cttgttaaca
7081 gggaacagaa catgataaat gtccagcaag cttgtctgct ccttcagctt ttcaaacgca
7141 gactgggtgca tttttatggc aggcaaatga caaaagaaaa agctgaattg ccctggcctc
7201 cagcttttcta tcagaaacag ggttaaagtg attaaagcaa tcattcaaga aagccctgcc
7261 gtttgtttac taaccttcat ccaacattta gctttgtagt ctacctgtga gaagatattt
7321 cagaagtatt agagataagg aaggaggatc tagcaaacca gtgaaaagag taggtgacca
7381 gttataaaat gctttccatg cacattgaaat gccaggcgaa cctatttctg ttattccagc
7441 agacaatcag cagtggctct agattattaa catattttcc tttcatgtat aaattcaaat
7501 atgtaattct agtccaaagc attctgtggc tggtaagcac atacttctg atttcaata
7561 agaaaacata gcaagggaag gctccattaa acaagttgtt tctgccctta gtaattctct
7621 aaacaagata ggaagaaaaa gtggacagta gtggagtatt aatagtgtgc tcttttcat
7681 ctctaaagca cgagtaagta agcgttcaaa ctactctgtg gtgggcatatc atttagagcg
7741 ctgtgaatga accactgctg ttctgccata ctttaatttat ttatattatt atttttattt
7801 tatttgtgtt ttatgtatt ttactaatta ttattttata ttactaattt attttctcaa
7861 tttaaatcct gttgcattca attttaatta cagtttttgt atctgccttc ccatacttgc
7921 taccacgctc cccattgcca ctgcggcctt atccatgttt tctgtgtaca ccactctcgt
7981 atcaccocag aataattatg agtgcacccc agacttttga aaccactaga gtcaacatgt
8041 ttgtctttga ggaaagccaa tgatgcttta gcatttttgg caggggtgga tgtgtgttta
8101 agtgggggtg gtgcagctcc ttattgtctg cctattctac tgttgttccc aatccacatt
8161 ccctgcgggg caccataacct gtgtgcattg caaagaattt ccgaccttca gagccagaag
8221 tgtttctcaa ttgatctctt ccagcctagg gttatagctg atgaattata atccttgcct
8281 tttccacacc tttacctggg cttaccatgg ccttaaaaca ttgcccaga atcagaattg
8341 tctcatgagt gagtggggca aggc aaatcc tgttccagac cagctgagaa tgtacctagc
8401 tgcagaagaa gtttagaaagt gtcattcttt acttatctac cagaactata ttcgaggtag
8461 attttagatt taaaaaaaaa gcaagttctc gtaggccttg aatccccccc ttgctatggg
8521 aaaatggatc attattataa tggactgtcc agtaaagtcc atgatttctc ctagacatgt
8581 tctctctctt tatgaacctg atcaagagtg atctctttaa gtcttttctt cataatcca
8641 cagcattttg tacttagatg tacttagaaa gaaccatata caggttagct catgattgat
8701 atgcaagcct tcaccactct acctgtccta aaagtcaggg acacaccttc ttcatttcat
8761 cagtccctac ttctatccag cattggcatc cagtaagtat tagtggaatg gacagacaac
8821 ccgaatttgt gctgatggca gtttaccctg ttttaactgt catccttctg ctactagaca
8881 tggatgagac ctgagacgat gggactgctc agaggctcct ggctcttgaa ctttagggca
8941 ccagaatccc ctgcagggtc tgagaaaaaca ggggtttctg ggccccaccc ccagagttcc
9001 tgattcctga ggtctggggg ggggcttgaa gatggacatg tttaacaagc tcccaggtga
9061 cgctggcaac tgctgcctca gggccatgct gagaacctc gccctacaca aacctttctg
9121 ggaaaaacaac tcaacattaa agctgtttgg ggatctctga agaaatctgt agtccctgcc
9181 ttgttggggg agcatcaggg atctaaccat tgatgggtga gtatttgttg ttaattcagc
9241 aagcaactat taagtgttag gcctgttact cggctctaac aatacaaggc agagtgaact
9301 gtaccctcga gatttaaagt ctaagtctc tagagagaag cccagggtggg agcaagcaca
9361 ttttagagtta ggtgcttggg gcaagggtggg gacacagaag aagggaatgg catttgcctc
9421 tggaggggtc cggaaacagc ctaggggagga ggagcttgag tcttgaaata ctgtgggcat
9481 ctctaagcaa agtcacagta gacagctgaa ataaagaaaa tagtaagcaa gccaaagaaa
9541 cagtatttca gccaaaggga gcgtgtgtct atcaagcca cctgtgaaca cgtcccagga
9601 ttctctgcat ccggccattg ctcaagacag atccctcaca ggaacagcta agccactgat
9661 ttcagctacc tgttcacgtg agaattatca gtacctactg cttttcaaaa tgagtatgat
9721 catggatagg tgaggcaatt cagtttcgca gagacagtag ggcaagtgcc actgtagttt
9781 agttaagggc acatgcttta gagtttggct atgtgagtcc aatcccagtt tagccattta
9841 ttagctgggt agcttttagga gcagtagcct tagtgtctct cagttgtccc atctctataa
9901 tagggacaat aacataatag tgctgaataa aagagtaaca aaatttttgt caacatttaa
9961 tgbattttaa gagctaagct ccgtgattgg cacaatgaac caatcaatca aacaccagtt
10021 gttattaata aaagtcaagt gaatatgtac tgtgtgctg gccgtgggtc aatttgcctt
10081 tgcatacaag gaaaaaatta aaatactctg ttaataaaga ctatagcata atactttcac

10141 cttaaacttc ttgatgttaa tttattttgt ttacctgcca aacttctact cattccttat
10201 gactttctgc tacatgaaac accctttgtt attcctttgt cctattaaat taagttctct
10261 ctcctctgct ttctgtcttt tgggtgcttt taataacact ttttaacctg gactttctca
10321 ttcagctgtg caactgtgga ctgagaggag gctctttgaa ttcattttgt atattctagt
10381 agagagtact gtgagcagtt ggggtgttga atgaatacat taattcaacc tggagggatg
10441 ggcagatttg cattttttac attgatatta catgatattt agaaaactgc ttaactgggtg
10501 gacgttggtt tattaacagc atttttgtgt tagcactcac tatgtgccag ctgctattct
10561 aactgcctga caaatactcc tgaaaccttc atggtaacca tatgaggga gcacttttaa
10621 tatatccata ataccaacgg ggagactgtg gccaaattgg ttaattaact tagccaaagt
10681 catattgaac taataagtgg atttaaaccc agctagtctg gggccagggg cctctttta
10741 atcttctgcc tctgtcttat gctgttgcat ggagtagtct ttatcatata actaaattaa
10801 gcatgcattt gcttaaagca gtgcatacat gatggatcaa aaagtgtgtg gtataattgg
10861 ttttaattctg tcattatcca ttttgattta tagtcacttt ctatgatgg tctgtagtgt
10921 ttaaattggaa cctttgaatc tttgatataa taaggttatg tcaaactctg ggtataataa
10981 ggttataccc aatggaaaca gaataatgat cagcccatth aaaggatgac tggagagtta
11041 ttacaatata taatagtcac gcataatttg agtagtattc ctttggtaac attttccttt
11101 taaaaattgt aacatttgat tgttcttgt tgggagaaaa ggaggtcaga tttttgaggg
11161 gagatccatt tgggtgagatg ctgagtggtg gtcaagctaa ggagatagta tgacatcttt
11221 tttagagtct agtcacaatt aaatgccatt ttattttgga ttttgggac cgtgccagct
11281 tccagcttgt cagagctgag aagactcaaa tcaagtccag gcttatttct acagcaaact
11341 gggattctgg ctctctgccc gtggattcat tcagtacagc ccatctggct tttgatgttc
11401 tgcaagtttg gagccatttg ttgaaggaag ccaggcgggtg aatatgtgtg gtccctgggt
11461 tctcttgact ccaagtgggt ccccttggtt tgcattttca ccatgcttag catctgctta
11521 cctggagacc atgcagccgc cggccagagg cctccaacaa ccaaactctc atgcctttta
11581 gaactcagag tccccagcac atccctcttc ctccctcttg tccaattact ttcagtcagt
11641 tctcagtagc tgettgtttg aatcacttat agtatttaac ttctaggggt tttttgggtt
11701 ttggtcaagg taattccagg ctgaatgtgg tgactaagca ggaaataaat gggctcgtct
11761 caaagttaca gtggagcgct gtttctattt tctaaggta cacagttgtg ggggcgatcc
11821 gtatggaagt caggaaccca gtctgatatt gcttctttt gatggtagca gtacagacct
11881 ggtgttttg tagcctgctt tgtttttctt cctttttctt cctaacttca cgggctgtgg
11941 caaagccctg agcgtgcag gaaaatgtct cctgtcatac gccacagca gacctagccc
12001 tgacctctct ctgaagccca ggaaggaggt atctgtgaag cagcctgctt gtaaagcaat
12061 tgcacacagc cttgtaaact gtgttactgg gctgattata cttgattggc aagggtgaatc
12121 tcttatagca aaagagaact tggagagttt tatctcatct tatgccttat taatttggtc
12181 attctttaat tacacagcca cctattgagc accctattta tgcaaggtag ctggctcggg
12241 gtcagagggg ggggtcccatg gtaaacgaga cagactcaat cctggaggag caggaatggc
12301 agccctctgc tgggtgttg gccccacaa aagggaagg tttcatttta ataatacatg
12361 ggtgaatcat tttgtcaat aggcataatt cttttagttt aaaaaaaaaa atagtggttag
12421 gaaggaaagg gatgggcaga ggggttaaac aaaagatag ctctccctaa ctctagattg
12481 tagtattgtt atgcttgtca ctgtagctga attccatttc tttgagtttt ttcaatgcca
12541 aggcattccc tgtatgactt acgtgagcct ttcactctcg cgatttttcc cattcaggta
12601 aatgagcaaa tggatttgaa cactcatatc taaaacaaga gagaaccagc tggaaatgcc
12661 ctttgaattt ctttctctat gtaaacatt tttctttctg gtgctcacc tataaataac
12721 aggagtcca ccttctttta tagactcttg ctgaaagcat ggtttggaac aagaccgtac
12781 aggtgcacac aaattacagt tgggaaagaa gcctgcagtg catcttgtct ctgaaggtta
12841 tgaaatcctc ctttttagta tggagctggc gtgatcaagc cagcaggatg aaatttggca
12901 tttgtgagat cccccctt ctacttgcc cactgtacat agcatcccag ccttactctt
12961 caaatctcca cattttttct tatctagcta caaaattcat aggtgattt ttttgggggtg
13021 cgtgtgtggt ttttttttg tttttttggt aaataaagac ctgcattttt attttgatat
13081 aggtggttga gttttgtctt taatttcatg acagagattt aactagtctc aacttttgaa
13141 aagacaacaa tgatatttg ggatcacaca cttaaagtta gatttctaga tgattaatac
13201 caaagtagat gattttttag cctcagccat ttataggat gcccttctgt gaatttttta
13261 tgacagtga aatcatggca cagataaaaa ttaataaat acttctgtta ttttctgaa
13321 gaaaaaaaaa aaaagcttaa actatgagaa tactgtcttt gagcacttta aaataaaatt
13381 gacttcagcc agcaggattt tgagcattac atcacaata aaaaacaaga ttaacatcaa
13441 aaggagtcag ttttcattca attgtgcagc actgtgggct gtgaaattta atattatttt
13501 gactcatatg ctaattgtag actgacagag gaaaatggat tgtgttttaa taaaaggata

13561 cacagcatca cagcagctg tatcaaatac aagttgaggt ctttgggcca ggaactgggg
13621 gccctctagc tctgttattg cagattcaag tttgacaaat aaaactttcc tttagactgt
13681 agtttaatta ctttttttca aaggatgctg tgatgaagag gcacaaatc acctcacctt
13741 gaagagttgc taaactgggt tgtgtgcca tcagttcacc gtgtgtttga atttctgtgc
13801 ttctcatctt tccttttctt gaaaagattt tgcttgcct tgggtgtgaat tgtaccccc
13861 acccccaccc atctagtctt tgctctcaga ttataacac tttaatgggt ccaaattgta
13921 tagcctgctc ttagacccct tttcttttcc ttgaataaat caggttcatg ttgcagacga
13981 tatttgtttt aggaaagtgt gaaagaagg gcacctgtga aaacacgcaa ttgttccaac
14041 acacatatac atccaaatta aagcagaaaa tgtcaaagcc tccaatcact acctatttcc
14101 ttggagggtt aaagccgctg agaagatagt ggtgccctcg ctggaagttt taaggtaatt
14161 actttttact ctaagcagta gtatctggta acctaattcc gtataaacct gacacctat
14221 cgctacaccc cagtatttct ctgatttcag aataagtcct cgtagaaact tgttctgatg
14281 ttaaagtgc aaggggggca gttaaagtgt atccacaaaa aaggaaaaac attttccaag
14341 tatttcttat tactgcctgt gtctttcgta ggccctgcct ttattttatc attttataac
14401 aaaactctta tgtttggggc attcagagaa taccttatta agctgttgca gcaatctagc
14461 attaaatgga agacatgcaa gactgaagat cctgcctgtt tatgaagtgt gccatcaaat
14521 tcacatgctc atgatgcaga gtccttcttt gggagtattc gtattcccaa gtgcacagag
14581 cacttcggaa aggagccttg gtctttgggt ttaatgctct cctagctccg tatagatgtg
14641 gcaggcccaa agtacatggt ggggtgaagg gctcagggtt tgggcttacc gagagcgcg
14701 tgcatccttt ctcaggaggt gactggaaac accagccaat tacagcagaa ctgcagactg
14761 ctcatctgca ttcggaattg cagatgaacc agtttgtact cgacttctct tcttactgt
14821 aggctttgac atttaattaa aaattaaagc cttttatgga aaaagtacat gttttccaaa
14881 atggggtaaa ttcgaagtat acttgataga gaacactggc ttgggaataa acctgtgata
14941 ttacatgact tttgggttgc aactgctagg ctgagcctct ttgtaaagct gggatttaga
15001 atctttgaaa tgtttgtaca gttcaatgat taagcataaa ttgtatatat tccctttttt
15061 tcacttattt gagtaaacaa gtttgttact acagcttctg tggactcaga tggttatgta
15121 ttaaatagga cacaacttca actaggataa ttttatttat ctgcttgtta gggaaattgca
15181 tcaaaagttt aagtctgtag gcattaaata ttttaaattg ttatttttaa agtcaattat
15241 gaaagatagc acaaagtttt tctgaaacta cattaaaaaa ataagtttt aatcttatca
15301 caaaagcatt gactatttat tgcaaagaaa acacagaaag ctaaaaatca ttctaagtcc
15361 accattcagt agcccaaagt ggtctcaggt aaaggcgggt tgtgtgacca tttgtttatg
15421 gttgtctccg tgcagtcagc aaaataaaca gaacaacatg ccataatata ttgatgtgta
15481 tattttcaac tgaaattagc catctgctta caatgatcat atacactaat ggttaatttt
15541 tgaaatgaaa agaaaaataa aataattctt tgtggagagt aatgcgaatt gacttatgaa
15601 tctcgccctg cttggcagtt tgctctagag gtagaagagc tttatgtgtg ggccctctcc
15661 cccccacac atttattctg ctccacttg caccagcatc catgtcagga ctaccttgt
15721 cctgttacat gagtaacatg gccctgattc tcaagtgcac gataactgcc ataattacac
15781 ataaatatta aatattttaa tagatcttta cgtgtgtaat attaggtaga agtggctctg
15841 gatcgaatct gatgcttttt aaatagaagc tttcccacaa catttccaag cactgtcatc
15901 gtgtctgtct cgatttgggg tttacctggc ctagtattct gtctgggtgt agaaactggg
15961 agttcctgtt tgtatctttt ttgttctgat ctctttatto tgtgtcagct aaatatctt
16021 gcagtcagtt actaacatat taactcatcc ttgtttggaa actttggcat atccttccat
16081 ggtttccttc cgtggacctg tcgcgtctct caggagagcc accaggtata ttgtcacaca
16141 tttcgcagatg attttcagag actacagcag catcaagtgg cccccagcg atttgggttt
16201 tcttctcggt taatctacac totttggcca accgtgagaa aacttgtaag aaggcatcag
16261 atgttttgtg taaggtgcgt gtagtatggt cagaggaaga aagaagcagg gaaaatggag
16321 tggccgtggg tgggagggga agcaggaggt gcaatttcgg gtccactaca cagctctcca
16381 taaacttctc cactgctggc tctcccagga tctcctatt acactgggca aagtgcagaa
16441 atagatcagg cgaccactgc ctccgtccat tcccaggca cctgtgaga cccgataatg
16501 caatacaggt cagcagaaaa gtccagactt gacatcccaa cgtgccatgg tctggtctgt
16561 gaatgaaaat cacatgaggt gacctctgaa ctctaagtgg ctggtttatg ttttcagtgt
16621 attaggcccg tgtttttaa aagcatgtgc tcgtagtgtg ggttaaaact ttctgtgtc
16681 ttcatttaatt atgctgtgtt ctagtctatt aatattaaag aatattgtgt tgcataatga
16741 ctaatttttt tatttttttg agacggagtc ttgctctgtc accaggctg gactgcagta
16801 gtgcgatctc ggctcactgc aacctccgcc tctcgatttc aagcaattct ctgtctcagc
16861 ctccagagtaa ctaggactac aggcgcccgc caccatgccc agctaagtgt tgtattttta
16921 atagagacgg ggttttacc tcttggccag gctggtcttg aactcctgac ctctgtatcc

Fig. 63(f)

```

16981 acccgccctca gcctcccaaa gtgctgggat tataggcggtg agccaccaacg cctggcaaca
17041 taaggactat. tttttaaaagt ttttacaatt atgactgtga agttgaaatg tctaaattat
17101 tagagatcca gttagatta ctaaataatt atgtctaatt gagatgatta gacttagcca
17161 aagtatccat gtagaagtat tagagtctag attgggtgaaa aacttgaaaa agcttggctt
17221 aagttcaata ggtaatccaa gagtaaaaac agattccaat atcagatctt ttcaccatag
17281 tcatgttaag tttggaagcc ctacttgagt gtttccagtt tttccacat tatatttgtt
17341 ctatatttga ttcaaaggca gggcatctat tgccttgctt aggactgatt cactgggaaa
17401 agccactgga gttgcctatt tccactcagt atgcctcact cttagagtag cttcccatgg
17461 ttcccaggca ggccctccag tgagaatgca ccaagccaca cgccatggcc tgggaagcag
17521 tccgaacct ggagattgtc ttgatgaaa ggaagaggca gccttccctt cccaggaaga
17581 tagtagagag cctgctctga cttcgctcag ggatggaact ggtctggctc agttctctct
17641 cctgtgtggg acatgaatca ctcttggtgg tctttgcttt ttatttgggc ttaaaatcag
17701 cagactttat taaatgacac ctctctctaa ccactctctg tctgggogaa gtttaacaag
17761 aacagccctcc ccccatgtgg tatgggttgt aactgtggcg gtttccctct gctgtttttg
17821 gttacaagat gaacattatc tgaacacaca gaaagaaatc tgtatttggc atccataatg
17881 gaaagggtgc ttagtaattt aaacttagcc agttatcctc atcataattc tttttaacac
17941 tttcaaagtc agcataggag aagtgatttg ttgaatatta caaaatattt agggcagaga
18001 tagatgtgct gtgtagtttg atttgttaat gtgtctaagc aatcaaagca acagaattca
18061 aatataaacc ccatcacttc caaaatagga actctgttta ctgacttgat tataacatat
18121 ggaactcaat tgttttccat taaaaaatga tactattagg aaactcacc cttttctttt
18181 tcatatatat tctgctattt gcataattgt ctggagtcca tatgtaatat taaatgtaaa
18241 acacaaatgc catgtagctg gtctgtttct tccctacctt ttggttccctg gcctcctggg
18301 gaagggttgc acatctgagc cgtggtctca gatgactgcc tcggaagaag cctcttccct
18361 tcaggcacca ctgatgtgtg cttggtgtgg agctagactt tccctggctc tccatgtgac
18421 gctcacatgt gcgtgtcttg atttccctta acttcatggc ttatctatga acagcttgat
18481 ttgggggaaa aaaatgtgtt tcccaatgct ggagttataa ttgaatgtgc tgcagtcaaa
18541 actgaaatgt gtgcagagaa agggggcctt tccctgtcatg ctcatgggc accagtgtgt
18601 cttcacctgt tttgtgtgtt aggtccatgc gtcatgctga aatgaagaac atgggatgta
18661 tggggctttg gacagtctg agccaaaagc aagtgtcaa aagcagctgt gtttgtatta
18721 ttagtgttgc tggaggtggc tgattgtcct gcattttaag tagagaggga ttgtagaaga
18781 ctgccaatac ttagaacttt tccagagag gaagggtcag aaactgcctc tgcaggtctc
18841 cttgctctcc agaaatgcc gtgtgcctgg gagggcatct tcagaaatcc agtctctcct
18901 cctcagtgtg tccgtgaccg actcagtggg tctgtcttca gaattcctat catgtctgtg
18961 atctgcaaat agtggtattt aatttgactt caatttgtat aaatgttagc ttctatttgt
19021 tcattcctat tttttgttca attaatacat tatttatgga gcatctactc tgtgtcagcc
19081 cttgggtgtt ttaactgta attagtaca tgtgggactt gcctgcccctc agggagctag
19141 actataaatt cctaattgatc agtggctctc acttttctgt cactcataat gtcctggcaca
19201 acataggttta cttgagttgt tacactcaca gtaactgtgt ttgctgccat ggtgctttag
19261 gaagtgtgag agttcccggg aggcagagtc aataatgcag actacacgta gtgaaaacat
19321 ggccaggaga gctgtagttc aggtctcag ctcaactgca ctctgtccac tgagaagcca
19381 taatttcttc acttaaagtg actgtgcgct atggctgttt atatatacgc ttaaaaagta
19441 aaagctgcta aaccactcaa ggattggggc cttttgtatt gatttaatta aaggaacaat
19501 cattgtttta atgagctcta gaaacaatta cttttgaaga gccgaggatc aaattcttgc
19561 ctcacgtttt gccacagtgt gttctgaaag gtgaattaat gcttttggaa tcatcaggaa
19621 tagtgagctt tgtcacgatt tactttttac aagcgtatct aatatgcata ttgaaatgtg
19681 agcctcccca ccacacttcc gctttgataa gcatcccccg gattgccgtc actgaccatt
19741 atagattttt aacaaagtgt gacagtacac actgaatgaa aactttacat caaggaaggc
19801 ctggcggtgt tgtaaaatga attaaaaggc tcattaaatg atttatatga cttacgcctt
19861 ctgaaaatat ggcccaaac acagagatcc ccaaagccac accgaccctt gcgtcccatg
19921 ttctcgacct caccgcatca gcaccagcaa gacctgtcgc tgagacggtg agtgatgaga
19981 gtcaagagga gtgacttgca tggcctggga ggaacacctc tgtgaatctt tagttaagca
20041 ggaaaaaaa atccctcatg aaggaaacag gatcttggga gcattttgaa tgaagaagga
20101 gcttagtgag ccaaacttga gacatagggt gtaatgtggg agagttttaa gatttgaga
20161 gatgtacagc ttgggagggg gtgtaatgca ttttctttaa agagctgaat gaatggttga
20221 ggaaatgggt acatctggtt tggttaagga tcctaactct tgaagcctgg gatgccccca
20281 gggcttgtaa tttaggaata ctccctctaa tagtagctaa cccttatata gtgctgtctg
20341 tgcaggctac aaaaggagca gattaaggat agaaaagggt tggagtgtat gagaaacctt

```

20401 aggccaggaat tgactcctgg tgtttgtaaa ccttaaagat gtccataaaa ggtcaaggaa
20461 taagacagga gaaaaaggaa atgtcaggaa gatgatcaat ttaatgttta tgggaatttag
20521 tttgtactta ctgcccgga tcttgctga ggtttttaac ctccagcagca catcagaatt
20581 actgtgtgtg tgttgagggg gctgggggag ataaagaaat tagcctcatc ccaaaccattc
20641 tgattcagtc tgttacttga gaaactgaat tgtgttttgt ccataaagaa gatgaaattg
20701 tctacagaga acacattgcc attcacaagg ttgaggggat accacagaga ggctcccact
20761 gtgatttgca tttgtcaaaa gttctagaga attcttcaac agtacacaca tgggtgtttt
20821 aaatatatca ttgttataaa aattcgtttt gagttctgtt tcacagaaag tttttttgaa
20881 tgaatgaatg tcatatatcc ttgctaaagg agctcagtta aaaaaaagg gaccatcctt
20941 ctcttttggg ggttgtacag taacacattc ccaagaaaga ggtaacagcc acatacattt
21001 ttcttcccaa taaagagtgt ggggttttaa tatgaatcca tagtatgatt tctgttatgt
21061 tttgtgctgc ttcataacca cactcatgca cttttcagaa aattaatacc attcattagc
21121 ataaatcata aactattccc ttggatggg tttgaaattg ggggtgccct atcatccttg
21181 ctttatctct tagtgaatta tgacctgta gtcactatgg ctgggtggcg tctctggtta
21241 aagaaagggt tggattggaa ggattcagag gcgattcttt gttcttaggc tttaatattt
21301 taatgagcct gcaggcttg ctgcttacga acgagctgag atttctaagt gtgtgttag
21361 tgttagcact tgtagaagga tgttcttag gaagttcttg tttcagtttt tcagagaaac
21421 tccccattaa gaaagatcat tcaggaaact gcctaccaag aaagaggaaa gggaggagg
21481 aggctttcag ctataagcat taaggggata ttgtatcagt agtcttagtt ctaaagattt
21541 gcttctgaga attaatgga gcaatacat ctcaaggga gaaaaaaaaa gatttatagg
21601 gcagggacag tagttgtcct tgcaagtaga ggacacttca ttttgcagct gaatcaatac
21661 cacaactaat tatttctggt tatcttttac gcatttgtaa gacattgctt ttgttcagtg
21721 taataaaaaa cccattgttt gatcagtgac tgactaatta tgataagtaa tttgaaacat
21781 tcttgatgaa acttgctgt taattaacat caacagcaca gggaaactaa caggacaaca
21841 aagtattagt ggatccactg ttccctccaa ttgacgagct ttctctgtgg catgccaat
21901 aaactaaagc tgccaatggt taaaaaataa caaacatgtg ggagatctga ctcaccacgg
21961 aggaagagtt atggtaaagt tacacaaagg agtactgaaa tattacaagc gagggggtgg
22021 taaagaaatg tcagcaggta gcctgatcct acagcttaga gtaaggaaaag tggtttcttt
22081 ctgtctttcc tttttctttt aaagcttaat tccaaaatac attcatccca tattgatctg
22141 aagtaagaga cttttgataa attaaagtgt gaactgaaa atgtgtagtt tgggattatg
22201 ggcattgcct ggctatcttg taactgtcat taactctgtt aatttttctc aactcaatgg
22261 cttttttttc ttatgctttt agattctac ttgacaaagg actgctacta taccacaga
22321 taccagacaa attggaatc acttgcctca aattggatc taaaaactgtt ggcagtagg
22381 atattctata agtttatatg ttgataaag accaaggaga cagatgcact attaagaagg
22441 aaataacccc tctctcaac tgtgcacaa cagaccaaga tctcaaatc accatcaagt
22501 tcaagaatt cagcctaac ctctgggtc tagaatttca gaagaacaaa gattattaca
22561 ttatatgtaa gtataatttt attcatttat tttatagaaa ttaagataag ctatataggt
22621 ttgtatcaat tttttgtttc cttaaaatta ttgtgacaaa taatttgatg aatatctatg
22681 tggaaaaaatt gtccccccc cctttttttt tttcaaagaa aacttcattg aatttgggac
22741 cctgtgctac cagtattcat taagtataca taccacaaaga gaaaaaaaaa cactagaatt
22801 cttaatagta ttgaaataaa tgtattatat gaatatattc agcatctcta ctgacaaaac
22861 catttttaag gaccattggt ggattttgat aggtaaatct tgtgcattgc cttttctctt
22921 caccatcca tccattcatt cactcattca tttcgtattt attctgtgcc agagactgtg
22981 cttaagggtc agggattcag cagtgaagg ttgtaaaata gcattgtttc ctcaagaagt
23041 taacagctta gagaagatgg agctcataaa ttcgaaagat ggggatgaca ggtcacatta
23101 aaaccagatt cagaagaaaa agcagaaact tggtttgctt agtacattac tcttttttgc
23161 atacatatat ataatttgac acgctgtttc aagaagagat ggtacgtatc ccttgggtca
23221 tatctgaggc tgacttgtga ggatgtgaag tcagctgatg agcacatttg gagccacgc
23281 ctactatgtg cagatctctc gtcagcgtca tcccagggc cccagggtgg gttaaagtct
23341 aggtgactca gacagctgtt cgcgtcattc aagcaatgaa gtcttttttc ttaatttctt
23401 tggtttaaaa ttatactcat aattaatgtg gttgaatttt ccagtggctt ggttaccata
23461 gacttcagtt tattaggga ctgctatctg ccactggttt attatttgcc ccaagggtga
23521 ctctaaaact ttaggtagga cactcttggt gatcaaaact aaactcttgc atctcaacct
23581 atgagccgca ctttattgtt attttatttt tttagagaca gggctctagt ttgttgccga
23641 ggctggcgtg cagtggcatg atcacagctc actgtagcct tgaactccag ggctcaagtg
23701 atcctccac ctccagctcc aagtagctcg gactacagge atgtgccact gcaccagct
23761 caagagctac acttcaaago acagaatgaa aacctatttt taaagccaac ttgatacata

23821 gagtagctta ccaagaatta gtaacaacaa caacaagaaa aaaaagagag aatgtggtag
23881 agtatatact tagtaaggag taattattat aaaataaaaag cattctgaaa tgaaacagggt
23941 agatgggggtg gccaggtatg cagcatagta gggaaatctt tgaaaatgta aaatagttac
24001 caggtaaaat aaatggaaac ttttaagcttt tggaagccta acaatgtatt tatattagta
24061 aagacttttat tttttttat tttttttat tttttttgag acggagtctc tctctttcgt
24121 caggctggag tgcagtggcg tgatctcggc tcaactgcaac ctccacctcc tgggttcaag
24181 tgattctcct gcctcagcct cccaagtagc tgggactaca ggtgtgcgct aatttttgta
24241 ttttttagtca agacgggggtt tcaccatggt ggccaggatc atctggatct cttgacctg
24301 tgatccttcc gccttgccct cccaaagtac tgggattcca ggctgagcc accgcgcctg
24361 gccttagtaa agacttttaa agtaagactt tttcagtga agctactggt aggcattgaca
24421 tttacaggca actgaaactg atcagatgca tttattaaga aggttaatgc ccttaggtg
24481 ggtgggagaa agaaggtcgt ggtacgggaa gaggggacac actagagatg agatgcccta
24541 gggcagtgaa cgcagtgtcc taatgcgtgg atgcagccca cgtccaccga taatgcgcac
24601 acaccagag tctctcttct tactttagct tatgacttca cgaagaatgc tttgcaaatt
24661 ctaagttcgc actgggcgca agtggaaatt tagtaaacat taagagttta accttagtg
24721 tgaaataata tgcaagatat gcaaatatt gtttaccac atctctttgc ttaatgtggt
24781 gagcatttaa taattgctt ttattaatac atgagagatt tgtatttaga agcagtttaa
24841 tttataatta taatattaat ctacacaata acgacatcta ttattttctt tttttggaaa
24901 ctcttcatac cacactaaca ggttcattgc agttactgaa ctactctggc catcagagct
24961 ctcttagag ttacgattta ccatgcaaaa gcatatggta gcctgggata aatgaatctt
25021 tcttaataca gaattgaggg tctcaagttt gaaactacga gaggctattt gaatgttgc
25081 ttgggggact gtcataaggg ctgggtggag gactcagggc taagaagttt gccaggaagt
25141 ccagttgaga ctttcagcag agttgaaaga cttccacgat ggctaggca gaggaaggcg
25201 tttcagatac ttgggaaaat atagaagcca atttctcacc caccctacag caaagctcat
25261 tgatctacaa gtttccttag aaaggaaatg ggaaatgcag agaacaaatg ttaaaatagt
25321 tttagaaatt aatattgact ttgtattgct tctgcataag ttccaagaca ccaaaacaat
25381 gaatggattt taaaaagtca ctactttgca tatcagacaa atgcacacac acacacacac
25441 acacacacac acacacacac acacacagtc aagctctgta ctggcttttt tgagaaggaa
25501 agtgtttgaa gtttagtaatt tttatatcag tacatttata aatagtgeta ggtagcatga
25561 cggaaagtat taaaatttac atgtattttt ttaacacttc aaatcgttgg ttcactttga
25621 gacagtaaat aatattagca tttgagttca gctttaataa attctacatg gggttaacct
25681 caaatctgag tgtctagttg gtaagcgctc tcagaacgag cagtgttata ataatatgt
25741 tattgtgtgc tgggttcttt ccatggagag gaaaaagaga cctgatgctt tggaggagt
25801 cttgactttt cccagtgag gagtagtcca gagggactga ctgcatgg ggagtacct
25861 acatgaacag catttcagaa gaattaaacc aggaacctag agtccactt gctagtctg
25921 cttcctaagg ttaatgagaa agtcaatttt atttctttga actttaattt atttccctaa
25981 aaaacgcttt tagtattgtc attgttctgg ctaatgatgg cggctcctc cagtttcaag
26041 ccaccttagg gctgggcata caaatgcaat ataggatcac ttgttagtgt ggtttcaaat
26101 ggacatgatc ctctgtaaat tctttaaaaa catttaattt gatttgggtt gttacctgct
26161 ttaaaatata gtcacacac ttgtgagttt cagacgtgaa tatgaatttt taatttgaa
26221 tgtattttta aacacactaa gtattaacta agtccctta ggagatatgt ggcaactga
26281 tatgcacctt cattcattct tctcatagat ggttatttgt tttttaactt gtggcaaat
26341 tatatatgaa tggtcaccga cttaaaatag ttccacttaa atttttcaac tttctgatgg
26401 gtttatttga gtattaaatg tattttcaat ttaatgatat tttcagotta ccttgtgctt
26461 atcaagtatc aagacatagc cccacctaa tcatggagca tctgtatatg ggttttattt
26521 cttgttttaga attgactttt tcaagtgacc tatttcagta attagccctg ggcctgattt
26581 gcataatgag atctcctaatt cttcaagtaa tgcaaagatg gagatattat ggccatgtgg
26641 tctgaagaga ccttttcttt attatgttca gatctttaat tgccttaaaa atagagtagc
26701 taatttacct aacctctagt tattttatta ttgtctttta agtttttttt aatgttcatg
26761 aaataactgt tctgaaattg cctattttca aggggaagctg tgtcttagac ttactaaatg
26821 ctccagttga tactgggaaa gccttcttgc gttcgtagcc tttatccgta gaggtttctt
26881 tgcagcattt tctgtgctg ttttagtttc ttttcagagg cgacaccag agctgaatga
26941 gtcagcaggt ttgggtgtgtc gaccctttgc aacagctgtc cttacgaagg ttctgtgggc
27001 tggttattct accttcgcat aaaaccttgc aaaataaccc acaaagaggt tttcgtcaca
27061 ctacccaaat catgtgagtc agagatggat gaaaaatgaa tgccattgtg ttcatacttt
27121 tccagtgaac agtagctaca gcagagctgt tagacaaaga aaaccgtatt aatgaagcgc
27181 ctcccaattt agcttcatat ggcttttgca ttattttgct gcaaatccat agctaagaca

27241 catcttgttg catagtccgt aagtcattctt tccgaaggac tgtttgatta aagggtgttc
27301 tgtgagatcc accctgtgtt gttcatggca tctcttggg ggccctccctc actctccatg
27361 ccttggcaaa gtcttcctta aggaacactg aacaagtctg gagaagctgc catttcttag
27421 ggccttcatt gggtcagttg tctatagctt tttattttttt attttttttt taataaagag
27481 tatgtaaaaat tggaaagctt cacaacacagc tttgtctattt tttagacatg tactccactt
27541 ctaagcaaaa tcacaaaata aagtaaaatg cttccacaaa tataatgaaa caatattctt
27601 aaagaatcaa agcagaagaa cttcagagtc tgttgcttat gttaagcata tatttgtttt
27661 cttctctgct tttgattttac ttatttctgg ggtgtaggtt tggcaagtag tactgaaacg
27721 tactgaatgc actgttcttt agcaagatag ttacaggagc tttcaaatgt cctcttaaca
27781 tatagatttc ttttagaata tagaataatg tgtgggctgt ataaagcgat tatgtgcttt
27841 atttgatgaa ttattttatgt acgataaatg tagcaaaagc cacatttcca tcattaaatg
27901 taatcccatt tgggtatata gcaacatcag cctgtcattt gggctcctctg attgaggggt
27961 gaggatttct gtttgatacc ttgtgcataa tggctgcgtt caagcattta aactcatttt
28021 tatttctaac ctacagctgt catctttgta ataggatatt catcagaatc ttgccagaga
28081 ctgtgcattt gggatcttgg gggatacagc accaccacca cctcccccct gtccaagaga
28141 aacagatcaa catcttaggt tgagagtctg gggctctggaa gacccgagtt cctgagtgc
28201 ctttgacaag taacttaacc cctgtctgcc tcagtctctt catctgtaaa gtggggataa
28261 tgacagcacc tgcttcacag ggttgatggg aatccagatg tgggtgggata tagaaaaatg
28321 ttattacttc cacctttgac accaaataga tataactaag agttaacttt ggagcagggg
28381 aggaagtgtg aggtccagg ctggaggcag acctgtgttc ggctgcaagc tggagaggat
28441 ggaccccaaa agcttggctg atttgaagtc catccataaa atggaactcc agagagttaa
28501 caggtttcag taatgctgca taacttaatt ataagatctt ctctctttgt cttctttcag
28561 tgttataaaa gctcttttgt ccttgagctt cctttacca gaaacatgca tttatgtatc
28621 tttttgttca tgggaattgcc caagcttgtt agcagatcct ttgtaagacc caaaagagac
28681 agacagggga ggagtcttca gatacatata atcatttttc ccaatttcca tgttaccagc
28741 cttgccagga ctttttctca gttccctgtt acacaatgaa aatagtgtct ctttattgat
28801 aatttttagta gcatcctaatt gtggtataaa tcgtcttcca gagaagaaaa tgtgtcaggg
28861 ttgcgttatc actgaggcta gctgggaaag tagatcagcc cattagtctg ataattcgaa
28921 gcgttggtttc tgttatttct gaacatcatg tgaactcctt ttctgggtgt attaaaggtt
28981 ttcccagtggt gtgtcagtg gactcctgat tgaatttaatt atgaataaag ataaattctt
29041 tacattttaag gattaaagtc tcagcttctg ctttaacttga gattgcactg agaaactcct
29101 ggctctcggg tatagcggag tcacgacctg gggatgtctg tcccatatgg ctctgtgtgt
29161 aagaagaaaa agctgctgtg gacggagact ctgttcacat taaatgacat cacctaaaggc
29221 atcatgacag caagaattat ttagggaattg ctcagaataa aactgccttc attatttcat
29281 aaaatgtatc ttggtatctt tagcacctta tttatggctt tttaaaggtt cactgggatt
29341 tataaataat tggacaatgc tagagacctt gtacaagaat gaaagaggac aggotctctt
29401 ctttaataacc ttttaaacatt catcaggaag ataaaaactt aaagcaaaat aaaacacatg
29461 aaaatagcca agatgcacag accagacaag caaatactac ttttaacttat ttgtatagtt
29521 ctttaagagtc acatttgttc ctgaagtttc aaaatctcgg gctgagtgtt tgatcactta
29581 ggaagtgtt gtggccttca catactcttg tctcactttg aagtctagaa acacaggtct
29641 tagagcaatt tttatcactg tgagaaagct gaaacttagt gtgagtagct tagtacaatt
29701 cagttggcca tcaaatgtca gaaacaaaac tcagtccagg gccgctggac ccttaggccg
29761 gcgttggttag tttacaacag tgcctcctgg gtccaaacat ctaagtgcac atgtagcaat
29821 agtaaagata gtatgtatgc atacataaca catatgtaga gacagcagag tatacgtaca
29881 cacatgttgc atacatagca acagcagaga agctcatgaa ctataaagga tggactgtat
29941 gcttgtatca gacatttttg tactgacgct ttgtcatata ttgtgtaaca tataaccagc
30001 ttgcaatcat atgccccaa agttgaacta agaaaatcct acagggtact aggaaggaa
30061 ggccattggg aaaaggtggt tatagtgga atttgttagc tcttatgaat tttcttttct
30121 tttttagaca tactcttaatt tccatttttt caataaatct atactatttt gtgtttttat
30181 gtttagcaagt actttaagcc cctcaataga aagttgctac atcatatagt gattaaaaat
30241 aaaaatctct caaacataga agtagaggtg gtatgagact tcaaattccc ttagccaagt
30301 acaagtgcag cagttttgtt ggctggctgg ctgcatagaa ggactgatgg attggcagac
30361 cctcaagctg gagtgttaatt gatctcatta cagaggagcc aggtgggtg acagttgtgc
30421 tttgcaagtg gttttttgca ttggtgaagt agcccatttt gttgttctg atgttaaca
30481 ggggatgaag gtattctttt attggaacaa acgagggaat ttgctctgga tcttagagg
30541 atagaacatg tcccctggac ggaataaggt tcatgtgtag ggcaaattta gataggggca
30601 ccttattggg gttactactg gtctctagat ggtcaaagca aacaacatgt ccatctaagc

30661	tgtgatgtcc	atctaagctg	tgtgtgtcca	tgagagtgc	gcattttctc	ctctgcagtg
30721	ttgttatatt	ctaaactgtc	agcagacatt	aattcggtcg	ctgggtgaagt	cccaccgcct
30781	agagatgaac	tctgcctccg	atggatgttt	tccacttcag	tgccactcgt	ctcgcaatta
30841	ctgggtcatt	aatatcattg	catgcaatta	gtgacagtag	aaagagctag	aggggtgtgg
30901	gatgtgcacc	ctccccacca	tgaacttttt	actctgaccc	tttcccagct	agaccttttc
30961	gtatcttggc	aaggatattt	taatgattga	gactgtcaga	atcttcagag	caggcactgg
31021	attatgtgct	ggaaataatt	cactcaaaca	cctgcttctc	catggttcag	aatattttca
31081	ttagatatta	tcactatccc	ttccctggga	agtttcattt	ttaaaaatct	gatgcttaag
31141	tacagctaatt	atagacaata	gggaattatg	ttttatcttt	agaactctta	cattattctt
31201	ttctttaaaa	atgtgagctg	agtcattgct	attgcagtgg	tcactctggc	gcctattttt
31261	aaaacacaat	tcctctatct	tagtagattt	tggcccatat	taagcatatc	aagaatgact
31321	tttttttttt	caagacatgg	ggttttattg	ggggcttata	tacaaggaaa	gagagagtcc
31381	agtggcagtg	ggctggacaa	gatatccaca	tggccctgtg	gcagtgagct	gggcaggaaa
31441	actgcaactg	cttgcaaaaca	gcatgtagtt	catctatagc	attttcactt	aacaccacco
31501	agctaataac	ttccacctgg	caaccttcac	ttaatccaga	acttaggacc	tcgagtcctt
31561	gtacggccca	tgttccacag	gatgggcca	gggctcagct	gttctccta	gacaaggaat
31621	gactctccac	attggccact	cccggttctc	ctagctcagg	acacatatct	aggtgtgtct
31681	aaggctggct	cttctatgtg	aagttactta	ttcttttacc	attgactctc	atgttcccac
31741	tatattaagt	ttttctgaat	tactgtggca	ataagaaacg	gtcccttaaa	ttatactaga
31801	agaaaagctt	tttttttggt	ttgtttttta	ttttgaaatt	atgttaaatt	ttttttctta
31861	actgagagat	tccacctgca	taaatcgtca	taacttttaa	cagtaagatc	ttagacttag
31921	aaagtgatgt	ttttcctcaa	cagaatttat	taaaaatcaa	gacaccaagc	tggtccaaac
31981	aatagtttga	ggggaaataa	aataaacaac	tccataaata	atcttatggt	gttaaacatg
32041	ttctctagcaa	aacaaacaaa	caaaaaagtc	gggggttggg	ggaggtgcag	tttattgcca
32101	gtactgtctg	gtctttctca	gaaaagcgtc	agtgtacatc	actgagcctg	gacggttatg
32161	tttcttgatc	tataccccct	atgtgtacat	gtgcttgcac	gcacacacat	gtagacacgc
32221	acacatgtgc	acctgccatc	actttctgct	cttccgtctt	ttcactcttg	agtgtctgta
32281	gccagtagct	ttccaggtct	gtatagtcaa	agatacctat	ggccctgaat	gtcttcaactg
32341	attgctattt	gacattcata	cggtttttaa	tgggttaaaag	gctttatgct	aaagctgtga
32401	tagaatttct	cctgttctag	atgtggtggt	tattgcttta	ttttgtgact	tttctctcag
32461	tagattgacc	ttctccctca	gtgtccaaagc	ctcgcatagc	atgatggcac	ctgtaaactc
32521	agttctgtat	cctgggtatcc	tttctcttcc	caagtagaag	caattaagta	atatatgtca
32581	tcaaaacctt	ttaagtgcac	atacaaaaca	aatcaactta	ccaaactgct	tcaaagttgt
32641	tccatgttta	acactcttct	ttctgagctc	tgggtagaat	gtcctattat	tggtcatcat
32701	gaatatattga	aattaaagaa	ataaaaactgt	accattttct	ttaagagcat	ccatttgtac
32761	ttgataacat	cttcagtcac	atttcaatgc	tggcaaagag	gaggggagtt	ctaaactgtg
32821	actcaatttt	agaactctact	ttttccaaat	tattctgttt	agtgcagaaa	actaattaat
32881	agtgttgcat	agaaaagtca	ctgaagctaa	gccagttatt	acttcttaat	gcatgattta
32941	ctgctttaag	ttttcaaaac	acaaccatag	caatgtggta	ttaattcaag	tgattcttcc
33001	tatcatattg	aacgatattt	tcacgggtga	aaaactcaca	catcctacat	cactgatagt
33061	ttatacagtg	ttttagctgt	ggctccctgc	atgcaaaaata	agagttaatc	aaatgtcagt
33121	gagaaccatc	tcatacaagta	gagggcttgt	tttgttttaa	ttacttttgc	taagtataaa
33181	tttcttcttg	aaaataaatt	ctgggcccgg	cgcgggtggc	cacgcctgta	atcctagcac
33241	tttggggagg	cgaggcgggc	ggatcacgag	gtcaggagat	cgagaccaa	ctggctaaca
33301	ctgtgaaacc	ccgtctctac	taaaaataca	aaaaatgagc	cgggtgtggt	ggcgggctcc
33361	tgtagtccca	gctactcggg	aggctgaggc	aggagaatgg	cgtgaacctg	ggaggcagag
33421	cttgtggtga	gccaagatca	caccactgca	ctccagcctg	ggtgacagag	cgagactccg
33481	tctcaaaaaa	aaaaaaaagg	aaaataaatt	cttctgtatt	tttctttctt	caagtgaggc
33541	catttagggg	aaagtatacc	ataaaaacttg	ctctaagata	aggcaaattt	ggtattatag
33601	gatgaagtgc	tatgtgattt	gaagtaatgc	tgaatttttt	aaatatatta	aactaaacaa
33661	gaataatgag	gccctcggaa	agtcagattt	atattttctca	tttttctcat	tttaaagcca
33721	cagtgaaaaa	cacataaaaag	gaagaagtta	gaaaaaaaaa	tgaatgaaat	tttttttttc
33781	cttttgggcaa	attaaataga	tgttttctggt	tcagaagatt	ttattaatta	acttttaaaga
33841	aacagtcatt	tatttttggc	attcagtgaa	cactatcatt	tccatgttta	gaacttttct
33901	tctaagttag	catcttaaaa	gataactgtg	aaactcaagg	cattcaacta	cattaatttg
33961	agtttcagaa	attgaattct	tgttttctaga	gtacatagtt	tgaattgatg	tcagggtggt
34021	aaatagataa	atcttagctt	cctaggttgt	atattcacac	taattatttt	tttatcagcc

34081 ttctttatttt tcaacttacc ttattctttt tgtttttttg acactcagat ttgatagccc
34141 tgtggttagaa gaaaacagta atacagtttg gtttgttgtt gtgttttgtt ttattttaaa
34201 gtcacggctt tgctttccat gttgttactg gattatgctt tttttaattc ttcagtttgc
34261 caagataaca gtcttccgat cttcagaagt ctgtatcaag cttaaggaaa ctgatgtgta
34321 ggaagactcg cctaagaagt ccaaattagc aaggctagca tgtgaggaca tgctggaaaa
34381 gaatagttcc catagatatt gacagagaat gttcataaaa tgctacttgt tttgtggtta
34441 catgagagta acttgtgtcc agtgcagctg tatgtaaggg caacgttttt attctgacga
34501 ctctgtggtt ttcatgaccc tggatgctta tcatgtctct ctgttgact tcttcaacgg
34561 agttgatata aatacttgct tccaagtgtc catctgccct ctctccatc ctggcccat
34621 acaaatacgc tacattttta aataatttga aataccctca atagtattta tatttcttgg
34681 tgcattcattc tttccataag aactgtgata ccattattct gtaggatttt tttgtgctt
34741 cccgtttcac atctctgtgc cagtgtgacc catatatcgg tgcaaatcca gaagttagat
34801 tgtccatctg attagcacac tgttagcaat gtggttgact aaacacagcc aagatgtggg
34861 gctggagctt agcctcctgg gagcagagcg gtgaacatca gatgaagaca tgtgaaaatg
34921 gagtactact tctcttctct ggggatgggc taaaaagcac agccagaaat attcttggcc
34981 ttccagtctg ctttacagtt actcactggt tctctttttt ttctactca gataaccagt
35041 atactcttcc cagtgtactaa gaactgcaga taagtatagg tgcaaataga tggcaaacccg
35101 agatggcag ctgtgtggtt tcagatgtgc tgcagaactt ttagacgatg tgaacgcaag
35161 gaactttttt gctgtgagct aatctctacc cactggaaat taggcctgg ggggaacaat
35221 gtagtgactt ctatatactt actacatgca gtttagacccc tgaagcaaaa gcttttaaaa
35281 acaggctgta aaatgcccat gtatctttat taagcctatt ttccaactgg atagagaaat
35341 tttctggtaa tttttaaatt tgtaaagtct atttttttcc tgagccaagg gaaaaaaaat
35401 atctgggccc taaaagctta gttataacaa tgttattttt tctatctctg aatgattaaa
35461 tgtgatttca tttatgtagc aatactatga tttgtgctgc attagatcac gctgatagaa
35521 agatacaaaag aaaaactaag tataatgaac taacaattta ttttactct tctctaaat
35581 taaaaattcc cagtacattc aaatgaacaa tgaaaataat tgcagaattg tctcctgaaa
35641 tggaaataga ttttttttcc caagcattag caatttcttg ttatttttca aaatcagcca
35701 ctaagccttt cagagcttct tggtagctat tgcaggagaa atcagaatat taatcttgtg
35761 gttttatttc agagtctgct gccaggaagg aggtataatt gggataggag actttttttt
35821 tttagctgtg tcaactgttca aggagggggg tttggaacct cagcataaga attacactct
35881 gtgatgagga tgtagcaggg gagaagaaag gtgattttca ctatgggaag ctactttac
35941 atcaagtata aaatagactg aaatagcttt ctgatttgta atacttgtaa agtttacctc
36001 ctggagtttc agtttagtacc agtgtactaa ctgggttaaa acagttcatg gcacctaga
36061 tcatttctaa ctcatggcaa aaatctttcc tgggtggaacg tgtaactgta ttttaaatgc
36121 ccctttataa gcaaccaagt atttgggatg ttattttgat attagtagtg aatttttcag
36181 tatcttccag taccctttgc aagtccacagg ttgacttaaa aggaaaagaa gcaaaatgct
36241 gaatatagca gaaaaactgt ctgcattcag actgttcagc ccacttttgc tccccacgtg
36301 gcaagcacac tccccaaaac aagcaatagc ctgtggcttc agaggaaact acaaaaggcag
36361 catctgtaga ttttctctc ttcaactcta agacttgaat gtttccctct tccccacaca
36421 cttttttttt aaaccaagaa ataaaaaagt tttcactctt aaagggtgcaa agcagtttca
36481 ttcttatgca acacagcctt cctcctactg tcttatagtc tgtggatgtt aaattataga
36541 ttccaattga attttaatac tctagagatt ttacatttgt ggttgtcaag acccgtttt
36601 ggtaaaccta gggagctccg cacaaaagca ttgatattca gaaaaggcac tgacctacaa
36661 attaaaagaa aaaaaaatca aataatgtgc acctcttgtg cttccagttt gacaaagcag
36721 aagtcacag cagttttctc ctctgcagac gcagttctca attctattta caagtaactg
36781 ctctactgtg cctgtttttc tcttctgtat actcatttaa tttgttttct tttggatctg
36841 aatctttgac tgtcttttcc cctcaagat taaaataaat acatctgtat tctccctt
36901 tctttctgtg cactgccctt cagatctcat tttgtcattt ttcagcttag tgttgaaact
36961 tttagcaaca aaaagtcagt tacttacttt gagtaagtaa ctcaaagtaa gtttaacttg
37021 agtttgagtg cacttttgcg tgtaggttca tttatgtgct tttaaactat gagccagata
37081 gattccacct gaatgaagta aaccaaacat cctaattttt tagaaaaatc aaataggaaa
37141 cttttcactt ctttctatat gcagacatat ctgctctgaa atgggtccaga aataggatct
37201 attctcaaca attaattgaa gattatagct gggaggaaaag gatactttaa aatttagtca
37261 gctcatagaa actcatagtt tgaagcctct agaatgaaga actgaaaaaa atcacacacc
37321 catatttggg ggagggaaaa gggaaagagc agggatagca ttaggagata tacctaagt
37381 ggggcctgtc gtgaggtggg ggactggggg acatggcaca cgtatacata tgtaacaaac
37441 aaatgacgag ttaacaggcg cagcccacca

37501 ctgcacgttg tgcacatgta ccctagaact taaagtataa taaaaaaaaa ttttaatagc
37561 cccattaaat aattaaaaag atttttttta gattcacaga agtgtaaaaa atttttaggt
37621 tttttttttt ttaagctgtc tgcgtgaatag tttcttaatg gtctacaatg tttgtatcta
37681 caaacagata ctgtctgctt cttactaccc ttccaagaca agtattatta tggcaattat
37741 tgcacagttt cccgggaaaa atttatccac agttacagaa gaatgagatg caattgtgag
37801 actgtaaagt ttaagcaagc actcagagaa gcacagtgat atgtatgcac agaagaggca
37861 gtctttgttt tgaggaaaac agtgaaaagta aagttaattc aagaccacaa agacaagtaa
37921 ataagtgccct tttttttgta gttaatatata tttcagtgga atgcatatct ctaccataaa
37981 tgcataataga acttggtttgc tgacctactg tttggaaaac aaacaatccc attagaagaa
38041 tgtcttttggg atttattttt accagaaaaat caatcctttt ttcagtcctt tgcaaatgac
38101 agtggttaciaa gccaaagactt tgataatcag gtgaaaaatg gattttaaatt gcagaaatgt
38161 atatgaaaca cttttgttcc ttgccccttg aacttttaggg gaatgaaaat gtctagcact
38221 ctccaccttc ttttctctcc tggaaacttga actgtaattc aaagcctgtt tctcattaaa
38281 gtacctggca gcctatctct ttacagcttg agttacaaag ctattcagag acctcgctgg
38341 tctaaagaga cagaacaagg atgtgtttta atagagcata ggctgttgaa aaaaaaatg
38401 ctgaaaatgg taaaatgatt ctgctcttcc ttcactcct cactgctgag gtggagaggg
38461 aattcagttg gtgaacacca gcaagtggct ggtaaaagtc cccactttct ctocagggtt
38521 gccacaggac ccagaatgag tgggtgggcat gtgtgtgaac cctctattca gccagagttt
38581 tccccgaaca ggtagtttgg ttgaagaggt tgactaaggt tgacattggc agtaataaca
38641 cgtatgttct tctgatttac aaaacgatgg aggaaaaagg ggagattttg aagacctgat
38701 ttctgggata cttcttaagc atgcataagg ctgaaaaaag aagacaaggg ttgtgggagg
38761 ctccctggctc agtggtttaca gaacttggat gcttgacaaa cagagcgtca agctaattgt
38821 tcttgaaagca ggaatctgac agtgaggaa gcaggtgtgg ggggatgatt accacgtttg
38881 gaaatggctg cattaactat tttgctcttc tgagtttggc cccaaaagag tccatagact
38941 ttttgaaagga tgccatccct tttatttata gactaacatt aaatcagtc tttgtgaagg
39001 aaggagaaaag tgccataaata aatttggagt cagatagcat acgtgcggca gtgtttccga
39061 tatccatttc tctttatttc tttttctttt tctttttggc tttcagcatc cccatacttt
39121 cagaaaaact gtgactaaga gtgaattctt atttttcaaa ttgttttcag acatttcctg
39181 ttcatgttaa cttggcttat tgatttctctg atttttcttt atttttttgt tttgtccatt
39241 ttattttttaa tcagctacat caaatgggtc tttggagggc ctggataacc aggagggagg
39301 ggtgtgccag acaagagcca tgaagatcct catgaaagtt ggacaaggta aagaccatct
39361 gctgcttcat gaogccactg tgacctgggtg tagcccccag ctatgatggt gctaatgttg
39421 ccgatgcca ccttcattcg ctcttctttt tagttttcaa agcaaacctt tctgcacttt
39481 gagccactga cagatttctt caagtcaatg tactaagctt ttattggaga tctaagagtt
39541 aagatcagca aggtagaatg tctattgcca tagatagata gatagataga tagataatag
39601 atagatagat agatagatag atatttcttt ttaaaaagca aaacactttg gttcaaaatc
39661 aaaatatcca gaatgaaaac taaaagcttg tgagtttttg ctcatctctg aatcttgact
39721 acagaagagt tttgttcatt gtgacttttc caatatagat aacctattgt gcagaaagaa
39781 ataattattc ttctaattaa aaattgggtat agtagtcaat caacttgctc agttaaatgt
39841 aaatgtcatc tgcaatgctt tgccctgcaa atgcaagaat ccctatagtt tccacagatg
39901 gcctcacgtt ctaaacctct gaaataacta gtataacat tttgttttaa aagaaaaatt
39961 atattcttgt atttcacagt actttgcata aagactctta tgttcattgc tattcatgcc
40021 tgttgaaata tatatgcagc tccataagct agatattgtc agatgtctgt gccgtaatta
40081 atcatttgggt tttcatatag atgcaagttc tgctggatca accaggaata aagatccaac
40141 aagacgtcca gaactagaag ctggtacaaa tgggaagaagt tcgacaacaa gtccctttgt
40201 aaaaccaaat ccagggtataa cagcatgatc tgtgtgtatg gaggtctgtg ggtaccacat
40261 tcttagtagt atcttaaaag gttagggcaga gtctaaagac ttctaaccag ttaggattag
40321 ctggaagtta cagtgatcag gaatctttgc tgtcagtgag tcattattaa ttacactcaa
40381 taagaacaaa ataactcatt ccaatgaaag tcatatattc aaaggagtag agttcatgag
40441 ctgtaagtgc cagttattag aactactctg tcaggccaaa ggtttcattg gctgacattt
40501 tatcaagctg gttgtcaact ccagcttaaa gctgatgtta atgtatatgt aattaattgt
40561 ctaatccctc atctaattat atctaagcca cagaggggtt aattgatcct cttctaattt
40621 ttaaatggta acatttttaa atattgcata atagtatatt ttcaggtggg tatcgttatt
40681 ttgtttcaca ttttccatgt aaaagaaaat attaaacagg tccctgacaa aagtgtagaa
40741 taccagataa aattgtccgt cgttgacctt cgttttctta acagtcttgg aacaaatagt
40801 tctgtatttg ttaccatgct aatgaaggtt ttatagagta gctgttgagc agacatcagc
40861 agttttgtat taggattggt gtgtgcttgc ttggtcgttg tgcaaattta tgcgtctgcag

40921 caatattcca tccctttcca agagtcaagg aggggaagttg ttattttctaa ctttcaatga
40981 caagatgtgt caaattcttg tgacaaactg ataaatggat aatataatga tgccaggcag
41041 ttttttagtg cttaacattt gggctggcag tctgttcggt gtgagagttt ctgctgcctt
41101 ccaaataat ttttaagtgt aatcaaataa tacagacgag ttacgagctg aacattttcc
41161 caggccccct cactccttcc gcgttcccg gctgttctgt tctgccagga ggcagggtct
41221 ttcttttagaa ggcaggccct ttgaaggttt gcatgaaact ccttttctca aaggaggcgg
41281 aagagcaata ccacataaac gctcaccgct gacctggaga attggccact tccctttttc
41341 ttccctgccg ctgccccagg ctggctgaca cgggttagaa gatgaagcaa gatcaagggc
41401 tggctgtcac cgacagtctg tgctcttgct ggataatgat acaaaggaaa cctgtgtgct
41461 tgggagggtg gggaggtccc tcttagagat acctctcatt tctttttgct ttgagctctt
41521 agacaggtg ttggcgaggc aaagtccagc ttctagttag taataagcct ggctattttt
41581 tcacattttt aagggtcata aaagcagtcg gtctgcactg ggacagcagt aactatctct
41641 gaccttttct gtctccgct ctgcagggtc tagcacagac ggcaacagcg ccggacattc
41701 ggggaacaac atcctcggtt ccgaagtggc cttatttgca gggattgctt caggatgcat
41761 catcttcctc gtcctcatca tcacgctggt ggtcctcttg ctgaagtacc ggaggagaca
41821 caggaagcac tcgcccagc acacgaccac gctgtcgtc agcacactgg ccacacccaa
41881 gcgcagcggc aacaacaacg gctcagagcc cagtgcatt atcatcccg taaggactgc
41941 ggacaggtc ttctgcccct actacggaa ggtcagcggc gactacgggc acccggtgta
42001 catcgtccag gagatgcccc cgcagagccc gggaacatt tactacaagg tctgagaggg
42061 accctggtgg tacctgtgct ttcccagagg acacctaatt tcccgatgcc tcccttgagg
42121 gtttgagagc ccgctgtctg gagaattgac tgaagcacag caccggggga gaggacact
42181 cctcctcgga agagcccgct gcgctggaca gcttacctag tcttgtagca ttcggccttg
42241 gtgaacacac acgctccctg gaagctggaa gactgtgcag aagacgcca ttcggactgc
42301 tgtgcgcgct cccacgtctc ctctcgaag ccatgtgctg cggtcactca ggctctgca
42361 gaagccaagg gaagcagtg gtttgttgac ggtcagggctg tgagcatcct ggagggtgct
42421 ccaggatgcc acgctggaa gggccggctt ctgcctgggg tgcatttccc ccgagtgca
42481 taccggactt gtcacacgga cctcggtta gtttaagggt gcaaagatct ctagagttta
42541 gtccctactg tctcactcgt tctgttacc agggctctgc agcacctcac ctgagacctc
42601 cactccacat ctgcatcact catggaacac tcatgtctgg agtccctcc tccagccgct
42661 ggcaacaaca gcttcagtc atgggtaatc cgttcataga aatttgtgtt gctaacaagg
42721 tgccctttag ccagatgcta ggctgtctgc gaagaaggct aggagttcat agaaggaggt
42781 ggggctgggg aaagggtggt ctgcaattgc agctcactgc tgctgcctct gaaacagaaa
42841 gttggaaagg aaaaaagaaa aaagcaatta ggtagcacag cactttggtt ttgctgagat
42901 cgaagaggcc agtaggagac acgacagcac acacagtgga ttccagtgca tggggaggca
42961 ctgctgttta tcaaatacg atgtgcagga agaaaagccc ctcttcattc cggggaacaa
43021 agacgggtat tgttgggaaa ggaacaggct tggaggggaa ggagaaagta ggccgctgat
43081 gatataatcg ggcaggactg ttgtggtact ggcaataaga tacacagctc cgagctgtag
43141 gagagtcggt ctgctttgga tgatttttta agcagactca gctgctatac ttaticacatt
43201 ttattaaaca cagggaagc atttaggaga atagcagaga gccaaatctg acctaaaagt
43261 tgaagagcca aaggtaaac aggtgtgta tccatcatca tctgtgttat taaagaatcc
43321 ttatctataa aaggtaggtc agatccccct cccccagggt tctccttcc cctccgatt
43381 gagccttacg acactttggt ttatgcggtg ctgtccgggt gccagggtg cagggtcggt
43441 actgatggag gctgcagcgc ccggtgctct gtgtcaagg gaagcacata cggcagacct
43501 cttagagtcc ttaagacgga agtaaatat gatgtccagg gggagaagga agataggacg
43561 tatttataat aggtatata aacacaagg atataaaatg aaagattttt actaatatat
43621 attttaaggt tgcacacagt acacaccaga agatgtgaaa ttcattttgt gcaattaaat
43681 ggtcccaatg ctccagcgtt aaaaaacaaa attggacagc tacttctggg aaaaaacaaa
43741 tcattccaaa aagaacaata atgagagcaa atgcaaaaat aaccaagtcc tccgaaggca
43801 tctcacggaa ccgtagacta ggaagtacga gccccacaga gcaggaagcc gatgtgactg
43861 catcatatat ttaacaatga caagatgttc cggcgtttat ttctgcgttg ggttttccct
43921 tgccttatgg gctgaagtg tctctaga

FIGURE 64. EphrinB2, mRNA

```

1  gcgcggagct  gggagtggct  tcgccatggc  tgtgagaagg  gactccgtgt  ggaagtactg
61  ctgggggtgtt  ttgatgggtt  tatgcagaac  tgcgatttcc  aaatcgatag  ttttagagcc
121  tatctattgg  aattcctcga  actccaaatt  tctacctgga  caaggactgg  tactataccc
181  acagatagga  gacaaattgg  atattatttg  ccccaaagtg  gactctaaaa  ctgttggcca
241  gtatgaatat  tataaagttt  atatggttga  taaagaccaa  gcagacagat  gcactattaa
301  gaaggaaaat  acccctctcc  tcaactgtgc  caaaccagac  caagatatca  aattcaccat
361  caagtttcaa  gaattcagcc  ctaacctctg  gggctctaga  tttcagaaga  acaaagatta
421  ttacattata  tctacatcaa  atgggtcttt  ggagggcctg  gataaccagg  agggaggggt
481  gtgccagaca  agagccatga  agatcctcat  gaaagtggga  caagatgcaa  gttctgctgg
541  atcaaccagg  aataaagatc  caacaagacg  tccagaacta  gaagctggta  caaatggaag
601  aagttcgaca  acaagtccct  ttgtaaaacc  aaatccaggt  tctagcacag  acggcaacag
661  cgccggacat  tcggggaaca  acatcctcgg  ttccgaagtg  gccttatttg  cagggattgc
721  ttcaggatgc  atcatcttca  tcgtcatcat  catcacgctg  gtggtcctct  tgctgaagta
781  ccggaggaga  cacaggaagc  actcgccgca  gcacacgacc  acgctgtcgc  tcagcacact
841  ggccacaccc  aagcgcagcg  gcaacaacaa  cggctcagag  cccagtgcga  ttatcatccc
901  gctaaggact  gcggacagcg  tcttctgccc  tcactacgag  aaggtcagcg  gggactacgg
961  gcacccgggtg  tacatcgtcc  aggagatgcc  cccgcagagc  ccggcgaaca  tttactacaa
1021  ggtctgagag  ggaccttgg  ggtacctgtg  ctttcccaga  ggacacctaa  tgtcccgatg
1081  cctcccttga  ggggtttgaga  gcccgcgtgc  tggagaattg  actgaagcac  agcaccgggg
1141  gagaggggaca  ctctctctcg  gaagagcccg  tcgcgctgga  cagcttacct  agtctttag
1201  cattcggcct  tgggtgaacac  acacgctccc  tggaaagctg  aagactgtgc  agaagacgce
1261  cattcggact  gctgtgcgcg  gtcccaagtc  tcctcctcga  agccatgtgc  tgcggtcact
1321  caggcctctg  cagaagccaa  gggaaagcac  tgggtttgtg  acgagagggc  tgtgagcatc
1381  ctggcagggtg  cccacaggatg  ccacgcctgg  aagggccggc  ttctgcctgg  ggtgcatttc
1441  cccgcagtg  cataccggac  ttgtcacacg  gacctcgggc  tagttaaggt  gtgcaaagat
1501  ctctagagtt  tagtccctac  tgtctcactc  gttctgttac  ccagggtctc  gcagcacctc
1561  acctgagacc  tccactccac  atctgcacat  ctcacgggaa  actcatgtct  ggagtcacct
1621  cctccagccg  ctggcaacaa  cagcttcagt  ccatgggtaa  tccgttcata  gaaatttgtt
1681  ttgctaacaa  ggtgccttt  agccagatgc  taggctgtct  gcgaagaagg  ctaggagttc
1741  atagaaggga  aagttggaaa  ggaaaaaaga  ggcctgcaat  gcagctcact  gctgctgcct
1801  ctgaaacaga  aagttggaaa  ggaaaaaaga  ggcctgcaat  gcagctcact  gctgctgcct
1861  ttttgctgag  atcgaagagg  ccagtaggag  acacgacagc  acacacagtg  gattccagtg
1921  catggggagg  cactcgctgt  tatcaaatag  cgatgtgcag  gaagaaaagc  cctcttcat
1981  tcgggggaac  aaagacgggt  attgttggga  aaggaacagg  ctbggaggga  agggagaaag
2041  taggcccgtg  atgatataat  cgggcaggac  tgttgtggta  ctggcaataa  gatcacagc
2101  tccgagctgt  aggagagtgc  gtctgctttg  gatgattttt  taagcagact  cagctgctat
2161  acttatcaca  ttttattaaa  cacagggaaa  gcatttagga  gaatagcaga  gagccaaatc
2221  tgacctaaaa  gttgaaaagc  caaagggtca  acaggctgta  attccatcat  catcgttgtt
2281  attaaagaat  ccttatctat  aaaaggtagg  tcagatcccc  ctccccccag  gttcctcctt
2341  cccctccgga  ttgagcctta  cgacactttg  gtttatgogg  tgctgtccgg  gtgccagggc
2401  tgcagggtcg  gtactgatgg  aggtgcagc  gcccgggtgt  ctgtgtcaag  gtgaagcaca
2461  tacggcagac  ctcttagagt  ccttaagacg  gaagtaaatt  atgatgtcca  gggggagaag
2521  gaagatagga  cgtatttata  ataggtatat  agaacacaag  ggatataaaa  tgaaagattt
2581  ttactaatat  atattttaag  gttgcacaca  gtacacacca  gaagatgtga  aattcatttg
2641  tggcaattaa  gtggtcccaa  tgctcagcgc  ttaaaaaaac  aaattggaca  gctacttctg
2701  ggaaaaacaa  catcattcca  aaaagaacaa  taatgagagc  aaatgcaaaa  ataaccaagt
2761  cctccgaagg  catctcacgg  aaccgtagac  taggaagtac  gagccccaca  gagcagggaag
2821  ccgatgtgac  tgcacatcat  atttaacaat  gacaagatgt  tccggcgttt  atttctgctg
2881  tgggttttcc  cttgccttat  gggctgaagt  gttctctaga  atccagcagg  tcacactggg
2941  ggcttcaggt  gacgatttag  ctgtggctcc  ctcctcctgt  cctccccgcg  accccctccc
3001  ttctgggaaa  caagaagagt  aaacaggaaa  cctacttttt  atgtgctatg  caaaatagac
3061  atctttaaca  tagtctgttt  actatggtta  cactttgctt  tctgaattgg  aagggaaaaa
3121  aaatgtagcg  acagcatttt  aaggttctca  gacctccagt  gagtacctgc  aaaaatgagt
3181  tgtcacagaa  attatgatcc  tctatttctt  gaacctggaa  atgatgttgg  tccaaagtgc
3241  gtgtgtgtat  gtgtgagtgg  gtgcgtggta  tacatgtgta  catatatgta  taatatatat

```

```
3301 ctacaatata tattatatat atctatatca tttttctgtg gaggggttgc atggtaacca
3361 gccacagtac atatgtaatt ctttccatca ccccaacctc tcctttctgt gcattcatgc
3421 aagagtttct tgtaagccat cagaagttac ttttaggatg ggggagaggg gcgagaaggg
3481 gaaaaatggg aaatagtctg attttaatga aatcaaatgt atgtatcatc agttggctac
3541 gttttgggtc tatgctaaac tgtgaaaaat cagatgaatt gataaaaagag ttccctgcaa
3601 ccaattgaaa agtggtctgt gcgtctgttt tgtgtctggt gcagaatatg acaatctacc
3661 aactgtccct ttgtttgaag ttggtttagc tttggaaagt tactgtaaat gccttgcttg
3721 tatgatcgtc cctggtcacc cgactttgga atttgcacca tcatgtttca gtgaagatgc
3781 tgtaaatagg ttcagatttt actgtctatg gatttggggg gttacagtag ccttattcac
3841 ctttttaata aaaatacaca tgaaaacaag aaagaaatgg cttttcttac ccagattgtg
3901 tacatagagc aatgttggtt ttttataaag tctaagcaag atgttttgta taaaatctga
3961 attttgcaat gtatttagct acagcttggt taacggcagt gtcattcccc tttgcactgt
4021 aatgaggaaa aaatggtata aaagggtgcc aaattgctgc atatttgtgc cgtaattatg
4081 taccatgaat atttatttaa aatttcgttg tccaatttgt aagtaacaca gtattatgcc
4141 tgagttataa atattttttt ctttctttgt tttattttaa tagcctgtca taggttttaa
4201 atctgcttta gtttcacatt gcagttagcc ccagaaaatg aaatccgtga agtcacattc
4261 cacatctgtt tcaaacgtgaa tttgttctta aaaaaataaa atattttttt cctatggaaa
4321 aaaaaaaaaa aaaaa
```


FIGURE 65. EphB4 Precursor Protein

```

1 melrvllcwa slaaaleetl lntkletadl kwvtfpqvdg qweelsglde eqhsvrtyev
61 cdvqrapgqa hwlrtgwvpr rgavhvayat rftmleclsl pragrsket ftfvyyesda
121 dtatalt paw menpyikvdt vaaehltrkr pgaeatgkvn vktlrlgpls kagfylafqd
181 qgacmallsl hlffykkcaql tvnltrfpet vprelvvpva gscvvdavpa pgpspslycr
241 edgqwaeqpv tgcscapgfe aaegntkcra caqgtfkpls gegscqpcpa nshsntigsa
301 vcqcrvgyfr artdprgapc ttpsaprsv vsrlngsslh lewsaplesg gredltyalr
361 crecrpggsc apcggdltfd pgprdlvepw vvvrgrlpdf tytfevtaln gvsslatgpv
421 pfepvnvttt revppavsdv rvtrsspssl slawavprap sgavldyevk yhekgaegps
481 svrflktsen raelrglkrg asylvqvrar seagygpfgq ehhsqtlde segwreqlal
541 iagtavvgvv lvlvvivvav lclrkqsngr eaeysdkhgq ylighgtkvy idpftyedpn
601 eavrefakei dvsyvkieev igagefgevc rgrlkapgk escvaiktlk ggyterqrre
661 flseasimgq fehpnirle gvvtnsmpvm iltefmenga ldsflrlndg qftviqlvgm
721 lrgiasgmry laemsvvhrd laarnilvns nlvckvsdfg lsrfleenss dptytsslgg
781 kipiwr tape aiafrkftsa sdawsygivm wevmsfgerp ywdmsngdvi naieqdyrlp
841 pppdcptslh qlmldcwqkd rnarprfpqv vsaldkmirn paslkivare nggashplld
901 qrqphysafg svgewlraik mgryeesfaa agfgsfelvs qisaedllri gvtlaghqkk
961 ilasvqhmkx qakpgtpggt ggpaqqy

```

FIGURE 66. EphrinB2

1 mavrrdsvwk ycwgvmlvlc rtaisksivl epiywssns kflpgggglvl ypqigdkldi
61 icpkvdsktv ggyeyykvyv vdkdqadrct ikkentplln cakpdqdikf tikfgefspn
121 lwglefqknk dyyiistsng slegldnqeg gvcqtramki lmkvgqdass agstrnkdp
181 rrpeleagtn grsstdspfv kpnpgsstdg nsaghsgnni lgsevalfag iasgciifiv
241 iitlvlll kyrrhrkhs pqhtttlsls tlatpkrsn nngsepsdii iplrtadsvf
301 cphyekvsd yghpvyivqe mppqspaniy ykv

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
23 September 2004 (23.09.2004)

PCT

(10) International Publication Number
WO 2004/080425 A3

(51) International Patent Classification⁷: **A31K 38/00**,
C07K 5/00

(21) International Application Number:
PCT/US2004/007755

(22) International Filing Date: 12 March 2004 (12.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/454,300 12 March 2003 (12.03.2003) US
60/454,432 12 March 2003 (12.03.2003) US

(71) Applicant (for all designated States except US): **VAS-
GENE THERAPEUTICS, INC.** [US/US]; 1929 Zonal
Avenue, Los Angeles, CA 90033 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KRASNOPIEROV,
Valery** [RU/US]; 346 South Los Robles Avenue, Apt.6,
Pasadena, CA 91101 (US). **ZOZULYA, SERGEY**
[RU/US]; 255 S. Grand Avenue, Apt. 2308, Los Angeles,
CA 90012 (US). **KERETESZ, Nathalie** [CA/US]; 4240
Lost Hills Road, Apt 1603, Calabasas, CA 91301 (US).
REDDY, Ramachandra [US/US]; 2101 Charlotte Street,
Apt. 21, Los Angeles, LA 90033 (US). **GILL, Parkash**
[US/US]; 1929 Zonal Avenue, Los Angeles, CA 90033
(US).

(74) Agents: **VINCENT, Matthew, P.** et al.; Ropes & Gray
LLP, One International Place, Boston, MA 02110-2624
(US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

(88) Date of publication of the international search report:
7 July 2005

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

(57) Abstract: In certain embodiments, this present invention provides polypeptide compositions, and methods for inhibiting Ephrin B2 or EphB4 activity. In other embodiments, the present invention provides methods and compositions for treating cancer or for treating angiogenesis-associated diseases.

WO 2004/080425 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07755

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A31K 38/00; C07K 5/00

US CL : 514/12, 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,624,899 (BENNETT et al) 29 April 1997, see columns 2-3 and 10-14.	1-11, 20, 22
X	US 5,635,177 (BENNETT et al) 03 June 1997, see columns 2-3	1-11, 20, 22



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

03 March 2005 (03.03.2005)

Date of mailing of the international search report

18 MAY 2005

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (703) 305-3230

Authorized officer

Sheela J. Huff

Telephone No. 571272-1600

Sheela J. Huff

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07755

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 23 and 36
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 23 is a duplicate and claim 36 refers to the polypeptide of claim 25 and claim 25 is directed to antibodies.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-11,20,22 and 34

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(2)) (January 2004)

BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-11, 20, 22, 34, drawn to polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, a composition and kit containing said polypeptide and a method of inhibiting signaling using said polypeptide.

Group II, claim(s) 12-19, 21, 24, 35, drawn to drawn to polypeptide comprising an amino acid sequence of an extracellular domain of an EphB2 protein, a composition and kit containing said polypeptide and a method of inhibiting signaling using said polypeptide.

Group III, claim(s) 25-33, drawn to antagonistic antibody, composition containing said antibody.

Group IV, claim(s) 37-49, 56 58-59 drawn to method of reducing the growth rate of a tumor.

Group V, claim(s) 50-55 and 57, drawn to method of inhibiting angiogenesis.

Group VI, claim(s) 60-61, drawn to method of identifying a tumor.

In addition, with Group III, applicant will have antibody to EPHB4 examiner without paying additional fees.

In addition, if applicant pays for Group IV, they will get Group IV as it reads on a polypeptide comprising an amino acid sequence of an extracellular domain of EPHB4 protein. If applicant wants any more peptides or antibodies, they need to pay additional fees.

In addition, if applicant pays for Group V, they will get Group V as it reads on a polypeptide comprising an amino acid sequence of an extracellular domain of EPHB4 protein. If applicant wants any more peptides or antibodies, they need to pay additional fees.

In addition, if applicant pays for Group VI, they will get Group VI as it reads on expression of EPHB4 protein. If applicant wants any more proteins/nucleic acids, they need to pay additional fees.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the products of Groups I-VI different structurally and functionally and this lack the same or corresponding special technical feature. Each of the methods in Groups IV-VI require the use of different products. The product in Group IV-V are polypeptides/antibodies whereas the product in Group VI is any agent. Groups VI and V do not have the same technical feature because in Group IV the polypeptide/antibody is used to reduce the growth rate of the tumor whereas in Group I it is to inhibit angiogenesis.

According to PCT Rule 13.2 and to the guidelines in Section (f)(i)(B)(1) of Annex B of the PCT Administrative Instructions, all alternatives of a Markush Group must have a common structure, which is a significant structural element. Although SEQ ID No. EphB4 and EphB2 share a common structure of a single amino acid, the compounds are not regarded as being of similar nature because the shared common structure is not a significant structural element. A common structure of a single amino acid is not a significant structural element because the amino acid is found in every amino acid sequence.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/07755

The number of inventions has been determined as follows: Each of Groups III has 1 additional sequences (in addition to SEQ ID No. EPHB4 . 1 X 1 results in 1 inventions. Each of Groups IV has 13 additional sequences (in addition to SEQ ID No. EPHB4 . 13 X 1 results in 3 inventions. Each of Groups V has 3 additional sequences (in addition to SEQ ID No. EPHB4 . # X 1 results in 3 inventions. Each of Groups VI has 5 additional sequences (in addition to SEQ ID No. EPHB4 . 5 X 1 results in 5 inventions. Thus, 1 + 3 + 3 + 5 plus the original 6 groups results in 18 inventions.

If no additional fees are paid, Group I, claims 1-11, 20, 22 and 34 will be examined.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.